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Mitochondrial DNA Analysis by Denaturing High-Performance Liquid Chromatography for the Characterization and Separation of Mixtures in Forensic Samples

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MITOCHONDRIAL DNA ANALYSIS BY DENATURING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE CHARACTERIZATION AND SEPARATION OF MIXTURES IN FORENSIC SAMPLES

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ABSTRACT

A mixture of different mtDNA molecules in a single sample is a significant obstacle to the successful use of standard methods of mtDNA analysis (*i.e.,* dideoxy dye-terminator sequencing). Forensic analysts often encounter either naturally occurring mixtures (*e.g.*, heteroplasmy) or situational mixtures typically arising from a combination of body fluids from separate individuals. The ability to accurately resolve and interpret these types of samples in a timely and cost efficient manner would substantially increase the power of mtDNA analysis and potentially provide valuable investigative information by allowing its use in cases where the current approach is limited or fails. Therefore, this research was aimed at developing a strategy for the use of Denaturing High-Performance Liquid Chromatography (DHPLC) as a developmentally-validated forensic application for resolving mixtures of mtDNA. To facilitate the adoption of this technology by the forensic community, a significant effort has been made to ensure that this technology meets the Scientific Working Group on DNA Analysis Methods (SWGDAM) developmental validation criteria and interfaces smoothly with previously validated methods of forensic mtDNA analysis. To do this, the method developed using DHPLC employs mtDNA amplicons, PCR conditions and DNA sequencing protocols validated for

use in forensic laboratories. These factors are essential in implementing DHPLC analysis in a forensic casework environment and for the admissibility of DHPLC and Linkage Phase Analysis in court.

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Chapter 1: The Introduction

§ 1 - 1 General Background

When traditional genomic DNA analysis based on short tandem repeat (STR) loci fail to yield results, forensic analysts have sought alternative means in order to determine a genetic profile. Mitochondrial DNA (mtDNA) analysis has often proved to be a powerful analytical approach to such forensic investigations. Conventional DNA typing relies on high molecular weight nuclear DNA, which can degrade quickly and thus fail to yield sufficient probative information to investigators. In these cases, the abundance, small size and circular nature of mtDNA make it an attractive target for forensic analysis. The scientific basis for mtDNA analysis have been thoroughly tested and validated by the forensic community and standard operating procedures are in place for sample handling, amplification and data analysis. The high copy number per cell (hundreds to thousands of copies/cell), subcellular sequestration and exonuclease-resistant circular nature aid in the successful analysis of severely degraded or otherwise challenging samples (Budowle *et al.*, 1999; Holland and Parsons, 1999). The small size of the amplification product obtained from mitochondrial hypervariable regions 1 and 2 (HV1 and HV2) also facilitates the analysis of challenging evidentiary material. This has been helpful in solving cases such as the identification of skeletal remains from the Vietnam War (Holland *et*

al., 1993); identification of bone material from a grave in Yekaterinburg, Russia, thought to belong to Tsar Nicholas II (Ivanov *et al.*, 1996); and the identification of shed hairs from robbery caps (Allen *et al.*, 1998).

Developments based on these and other studies have resulted in an accepted approach to mtDNA analysis that has withstood several court challenges (*e.g.*, State v. Council, 335 S.C. 1, 515 S.E.2d 508 (S.C., Apr 05 (The State v. Donney S. Council, 1999)) (NO. 24932), People v. Klinger, 713 N.Y.S.2d 823, 2000 N.Y. Slip Op. 20450 (N.Y.Co.Ct., Sep 05 (People v. Micheal Klinger and Raymond Klinger, 2000)) (NO. 0849/00), State v. Ware, 1999 WL 233592 (Tenn.Crim.App., Apr 20 (State of Tennessee v. Paul William Ware, 1999)) (NO. 03C01-9705CR00164)).

§ 1 - 1.1 Mitochondrial DNA Genetics

The two strands of the ≈16Kb human mtDNA genome differ considerably in their base composition. The heavy strand is rich in guanine and the light strand, rich in cytosine. A duplicated stretch of the heavy strand exists as an independent fragment (7S DNA). This fragment binds to the D-loop where it participates in the formation of a triple helical DNA structure. The sequence of the mitochondrial genome encodes a total of 28 genes on the heavy strand and 9 genes on the light strand. Of these, 22 encode mitochondrial tRNAs, two encode rRNA components (16S and 23S) of mitochondrial ribosomes, and the remaining

thirteen are templates for mRNAs that direct the synthesis of polypeptide subunits that contribute to the structure of mitochondrial respiratory complexes.

The mitochondrial genome contains very little non-coding sequence (7%), in contrast to the nuclear genome where approximately 97% of the sequence is thought not to be expressed. The 37 genes of the mitochondrial genome lack introns, are nearly contiguous with each other, and even overlap in some cases. With the exception of one or two non-coding bases that often exist between neighboring genes; the only significant non-coding region of the mitochondrial is the displacement loop also known as the D-Loop. This region of triplex DNA, also known as the "control region", contains the origin of replication for the heavy strand and the predominant transcriptional promoters for the generation of large multigenic transcripts. Portions of this region, designated hypervariable region 1 (HV1) and 2 (HV2), are of particular forensic utility because of the high degree of sequence polymorphism they exhibit within human populations. A third region, hypervariable region 3 (HV3), has also been described and is used only by a handful of laboratories and then only to try and differentiate indistinguishable HV1/HV2 samples (Lutz *et al.*, 2000; Bini *et al.*, 2003).

The mitochondrial sequence database MITOMAP (www.mitomap.org) which maintains a list of published mtDNA polymorphisms currently lists 845 polymorphisms (as of January 28th, 2011) that occur throughout the 674 bases of

the forensically informative HV1 and HV2 regions. Single base substitutions (56% transitions and 33% transversions) account for the overwhelming majority of these polymorphisms, although insertions and deletions are also seen. Even though many of these polymorphisms are seen only infrequently or are limited to specific ethnic populations, pair-wise comparisons of mtDNA sequences for both hypervariable regions between maternally unrelated individuals usually reveal multiple sequence differences. Based on studies of 4839 unrelated individuals the highest amounts of sequence diversity recorded are observed in populations of African extraction with an average of 14.5 sequence differences between individuals (Monson *et al.*, 2002). The lowest diversity was seen in Caucasians where an average of only 7.9 differences has been reported between individuals. Even so, the most common HV1/HV2 haplotype found in Caucasians account for approximately 7% of the population. Only 12 additional haplotypes are found at frequencies greater than approximately 0.5% (ignoring C-stretch polymorphism in HV2).

By some estimates, mutations arise and are fixed in the mitochondrial genome at a rate approaching twenty times that of equivalent sequences in nuclear genomes (Parsons *et al.*, 1997). A number of factors have been proposed to account for this degree of sequence instability including an inefficient system for DNA repair, oxidative damage, and the greater number of replicate cycles

that mtDNA undergoes relative to nuclear DNA during cell growth and development (Pinz *et al.*, 1995; Shadel and Clayton, 1997; Dianov *et al.*, 2001).

§ 1 - 1.2 Inheritance Issues

The mitochondrial genome is maternally inherited thus an individual's haplotype is a direct reflection of the up to 100,000 copies of the mitochondrial genome present in the oocyte at fertilization. The preponderance of evidence examined to date suggest that in most individuals, in spite of an accelerated mutation rate, the majority of mtDNA molecules within any single individual at maturity will still be represented by a single sequence (homoplasmy). Occasionally, a *de novo* mutation may arise and proliferate within the mtDNA population resulting in the existence of two distinct haplotypes within a single individual (heteroplasmy). This should theoretically, increase the discriminatory power of forensic mtDNA analysis by providing an additional character state for inclusion in the haplotype. This was the case for forensic analyses of mtDNA that were used to help identify the putative remains of Czar Nicholas II exhumed in 1991 from Yekaterinburg, Russia (Ivanov *et al.*, 1996). Haplotyping of bone fragments suspected of being those of Czar Nicholas revealed a rare heteroplasmic mixture of mtDNA at position 16169 in HV1 where C and T appeared to account for about 70% and 30% of the mixture, respectively. Haplotyping of mtDNA from the exhumed remains of the Czar's brother Grand Duke Georgij's revealed a similarly rare heteroplasmic mixture of C and T at

position 16169. When combined with sequence identity throughout the remainder of the D-loop, the inclusion of a shared heteroplasmy significantly strengthens the weight of the mtDNA match. In practice, however, the overlapping peaks seen in direct sequencing data from the PCR-amplified mtDNA of heteroplasmic individuals often complicates interpretation of the electropherogram. In cases of near equal mixtures of mtDNA, such positions are often discarded by the analysis software or the analyst as ambiguous bases. In other cases, the position may be subjectively called by an experienced DNA analyst. The exclusion of a base from consideration results in a decrease in discriminatory power.

§ 1 - 1.3 Mutation Rates

Due to the lack of recombination and the lack of highly effective DNA repair mechanisms, mtDNA provides an excellent opportunity to investigate human evolution and migration. Mitochondrial genetics, due to strict maternal inheritance, enables researchers to gain a higher resolution perspective on modern human population structures and divergence times (Cann *et al.*, 1987; Ingman *et al.*, 2000) than would be possible based on nuclear markers. More significantly, this makes it possible to divide humans into a collection of mtDNA haplogroups which can be used as biogeographic indicators of genetic populations.

The high rate of nucleotide substitutions in mtDNA has been of interest in the scientific community for some time. Various contributing factors have been proposed to explain this. These include the oxidative environment of the mitochondria, and insufficient repair mechanisms (Shadel and Clayton, 1997). Poor DNA replication activity could also be raised as a contributing factor, since only one DNA polymerase is dedicated to mtDNA replication. While polymerase gamma (γ), a high fidelity polymerase, with 3'-5' exonuclease activity (Copeland and Longley, 2003), would not be predicted to result in high mutation rates, oxidative damage can cause misincorporation during DNA replication (Pinz *et al.*, 1995). Additional DNA repair mechanisms include nucleotide excision and base excision repair. Nucleotide excision repairs (NER) restores the majority of UV-induced DNA damage (*e.g.* thymine dimers) by removing a short section of single stranded DNA surrounding the lesion. This is subsequently filled in by DNA polymerase using the opposite strand as a template. Base excision repair (BER) restores the integrity of DNA bases that have been modified by deamination or alkylation, causing incorrect base pairing. This is achieved by removing the modified base to yield an abasic site. This is subsequently filled in by DNA polymerase much as is the case for NER (Friedberg *et al.*, 1995). While BER appears to be efficient in the mitochondria, NER is not as well developed (Clayton *et al.*, 1974; Dianov *et al.*, 2001).

Human mtDNA recombination and the possibility of paternal inheritance have been topics of discussion in recent years. There is a lack of evidence supporting recombination of human mtDNA and periodic reports in the literature claiming recombination of human mtDNA have been strongly criticized as being due to sequencing errors, incorrect phylogenetic construction due to assumptions of hypothetical ancestral haplotype extinction and rejection of mutational hot spots (*i.e.,* sites within the mitochondrial D-loop which mutate even more rapidly than others in the same region) (Budowle *et al.*, 2003). The inability to even detect recombination of mtDNA has been largely unsuccessful, even in experiments where hybrid cells are formed from two donors (Shadel and Clayton, 1997). If, in fact, recombination does occur, it would appear to be extremely rare. The same can be said for paternal inheritance of mtDNA. Initial claims were reported by Hagelberg *et al.*, where a rare mutation was detected in three unrelated populations within one geographic origin. This could only be explained by paternal leakage and recombination. The paper was later retracted due to sequencing alignment errors, but the argument continues as reports of paternal mitochondria entering the egg have periodically appeared in the professional literature (Cummins, 2000). Even in cases where this might occur, however, the paternal mtDNA sequence would be rapidly and enormously diluted since unfertilized eggs have been reported to have as many as 100,000 mtDNA genomes (Chen *et al.*, 1995). Furthermore, selective destruction of sperm

mitochondria by the developing zygote due to ubiquitin tagging which takes place during spermatogenesis would appear to effectively eliminate the potential for paternal leakage (Sutovsky *et al.*, 1999).

A single case of paternal leakage has, however, been reported in the literature (Schwartz and Vissing, 2002). In this case, the subject suffered from mitochondrial myopathy as a result of a novel 2bp deletion in the gene encoding for a subunit of the enzyme complex I of the mitochondrial respiratory chain. It could be that the pathogenic mutation conferred a selective advantage allowing it to escape destruction. Additional studies of individuals suffering from the same muscle disease have, however, failed to identify any evidence of paternal transmission of mtDNA (Filosto *et al.*, 2003; Johns, 2003; Taylor *et al.*, 2003).

§ 1 - 2 Forensic Assessment and Validation Standards

Ever since scientific evidence became an integral part of the criminal justice system a question of whether the evidence is trustworthy and based on accurate and consistent scientific methods has been asked. In order to be admissible to a court of law, any item of evidence must be obtained, presented and analyzed in a manner that is generally accepted in the scientific community. Ever since 1923, following a decision by the District of Columbia Court of Appeals, and subsequently referred to as the *Frye* standard (Frye v. United States, 293 F. 1013, 1014 (D.C. Cir. (Frye vs. United States, 1923)), a technology

or methodology cannot be considered admissible until it has been widely accepted by the relevant scientific community. The *Frye* standard, however, was and still is criticized for being overly restrictive, as it can dismiss testimony based on novel but credible scientific approaches which have yet to gain wide acceptance as measured by the professional literature. As the speed of scientific development increases, this imposes a significant obstacle for newer technologies.

As a result of *Daubert vs. Merrell Dow Pharmaceuticals, Inc.* (509 U.S. 579 (Daubert vs. Merrell Dow Pharmaceuticals, Inc., 1993)) the rules of general acceptance have been broadened such as to allow for admission of scientific technologies and resulting evidence that are lacking in broad acceptance in the scientific literature. Known as the *Daubert* standard, admissibility requires that the technology rest on sound scientific footing; that it has been thoroughly tested and validated; that it has representation in the peer review literature and that it has an established error rate. The change to the Federal Rules of Evidence in 2000 cemented the evolving *Daubert* standard, bringing into practice rules that not only outlined the standards associated with the evidence, but also the testimony of expert witnesses based around that evidence.

Testability of the scientific methods must be obtained through extensive validation studies performed within the laboratory as well as between

laboratories. In terms of DNA validation studies, several agencies have published explicit guidelines on factors to be taken into consideration when performing them and the specific experiments to be conducted. Results of the validation studies are used to determine the error rates necessary for the method to be considered scientifically sound. Data from validation studies are then published in peer review journals.

The Scientific Working Group on DNA Analysis Methods (SWGDAM) has published revised developmental validation guidelines for the implementation of new forensic DNA analysis technologies (SWGDAM, 2004). For the purpose of mtDNA mixture analysis, previously validated procedures for mtDNA analysis on genetic markers, species specificity and amplification conditions were not repeated as they were not germane to the application being developed. However, reproducibility, sensitivity and accuracy of the mixture separation method in question were determined in accordance with the established guidelines and the previously validated amplification procedures and conditions.

§ 1 - 2.1 Current Approaches to Forensic Analysis of mtDNA

In contrast to the majority of forensic DNA profiling methods that rely on the identification of length polymorphisms in nuclear DNA, mtDNA profiling relies on direct examination of DNA sequence from an extra-nuclear molecule of DNA. With rare exception (Iwabe and Miyata, 2001), mtDNA is present in the

cytoplasm of virtually all eukaryotes. In humans, the mitochondrial genome is a single circular molecule of DNA typically consisting of 16,569 base pairs (Anderson *et al.*, 1981). The majority of human cells contain several hundred to thousands of copies of the mitochondrial genome such that the total compliment of mtDNA accounts for as much as 0.5% of the DNA in a nucleated cell. The abundance of mitochondrial molecules makes mtDNA an especially robust target for forensic analysis since it can often be amplified from tissues where sufficient amounts of chromosomal DNA may be absent (*e.g.*, hair shafts and environmentally degraded tissues).

The profiling of human mtDNA is achieved by comparison of DNA sequence data to the revised Cambridge Reference Sequence (rCRS) (Anderson et al., 1981; Andrews et al., 1999) (Figure 1.1). Differences are noted and reported by indicating the nucleotide position and the identity of the altered base. Automated DNA sequencing of PCR-amplified hypervariable regions of mtDNA is the current method of choice for forensic analysis. Amplification of the HV1/HV2 regions of the D-loop is achieved by use of up to four pairs of PCR primers (Wilson et al., 1995). For hairs without follicle tissue or for degraded tissues where severely fragmented or low levels of mtDNA are likely to be present, all four primer pairs are employed. Two primer pairs amplify HV1 to yield overlapping products of 278 and 271 base pairs, respectively. These primers pairs are designated L15997/H16236 (defining HV1A) and L16159/H16391

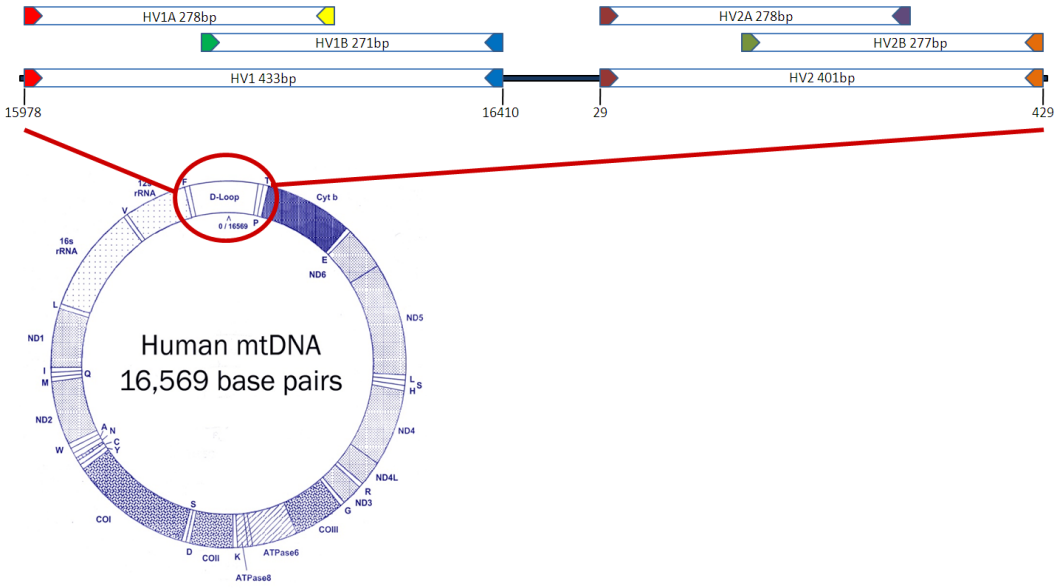


Figure 1.1: Schematic representation of the circular human mitochondrial DNA genome showing the locations of the D-loop and genes encoded by the circular genome. The D-loop region is divided into two large fragments, HV1 and HV2 (433 and 401bp, respectively) which for forensic purposes are further divided into two amplicons each (HV1A, HV1B, HV2A and HV2B). These smaller fragments are amplified using the primers listed in table 2.1.

(defining HV1B) where “H” and “L” refer to the heavy and light strand and the number indicates the base position of the primer relative to the Anderson sequence (Anderson *et al.*, 1981; Budowle *et al.*, 2000). Two other primer pairs (L048/H285 defining HV2A and L172/H408 defining HV2B) amplify the HV2 region to yield overlapping products of 278 and 277 base pairs, respectively (Figure 1.1).

Additional primer pairs have been developed and are currently in use in the forensic community. The Armed Forces DNA Identification Laboratory (Rockville, MD) has developed primer sets which include “mini-primers”. These are sets of amplification primers optimized for amplification of severely degraded remains. These further divide the mtDNA hypervariable regions into eight overlapping regions maximizing the potential to amplify target sites from highly fragmented mtDNA molecules (Gabriel *et al.*, 2001).

The PCR amplified fragments serve as templates for DNA sequencing by the dideoxy chain termination method (Sanger *et al.*, 1977). The DNA templates are cyclically denatured and nascent strands of DNA are synthesized in the presence of fluorescently labeled dideoxyribonucleotide terminators. The use of PCR and laser-induced fluorescence for sequencing makes it possible to analyze extremely minute quantities of evidentiary material.

Alternative sequence detection/screening technologies for the analysis of mtDNA have been investigated. In recent years, these include the use of immobilized sequence-specific oligonucleotide (SSO) probes (Reynolds *et al.*, 2000; Gabriel *et al.*, 2001), microarrays (Fukushima, 1999) and time-of-flight mass spectrometry (Butler and Becker, 2001). These approaches are limited to the detection of polymorphisms for which the researcher has *a priori* knowledge. Thus, it is often necessary to design additional probes in order to detect novel or population-specific polymorphisms.

§ 1 - 2.2 Mitochondrial DNA Validation for Forensic Casework

Validation studies have been performed for consistency in mtDNA profiling. These span a variety of tissue types, varying degrees of degradation and environmental contaminants as well as cross amplification testing between species (Wilson *et al.*, 1995). Sequencing results were obtained for known samples of hair and blood as well as blood and semen stains on various substrates. These substrates included fabrics, such as nylon and denim, carpet, leather, wallboard and wood, all of which represent frequently encountered types of evidentiary material. To test the detection capabilities of the amplification process with degraded material, tissue samples were incubated at room temperature for a number of weeks prior to DNA extraction. Sequences were also obtained from blood subjected to environmental contaminants

commonly encountered in a forensic context such as used motor oil, gasoline, soil, detergent, NaOH and acetic acid.

Additional validation studies tested the cross-reactivity of the human specific primers on non-human samples, such as rabbit, chicken, goat, horse among others, as well as several species of primates (macaque, orangutan, Celebes ape and gorilla). Macaque, orangutan and gorilla yielded amplifiable products; however, sequencing results on 215 bases from gorilla yielded 37 polymorphisms and 5 insertions when compared to the human reference sequence. For the same region, a database search of 1907 human sequences revealed that the greatest number of polymorphisms observed was 13.

Haplotyping of hairs subjected to a variety of treatments was performed as part of the validation study. DNA extracts were prepared from hair treated with conditioners, dyes and microscopic mounting media, as well as hair contaminated with dirt and body fluids. Hair samples were recovered from deceased individuals as well as from various areas of the head and body. Due to the rudimentary extraction procedures as well as insufficient knowledge of the ease of mtDNA cross-contamination at the time of the validation study, reagent blanks and negative controls frequently resulted in amplification at a level greater than 10% of the amplified product from the known sample. This was most likely due to reuse of grinding vessels employed during the extraction

process. For samples that were not contaminated, the haplotypes obtained matched the donor's blood sample haplotype in all cases. Current interpretation guidelines, however, still follow the 10% "contamination standard", where the presence of underlying peaks in the sequencing electropherogram are considered dye-terminator artifacts as long as they account for <10% of the major peak. Due to improvements in sample extraction protocols, cross contamination between samples has been significantly decreased from that reported in the original DNA extraction validation studies. For example, the contamination rate was determined to be 2.4% based on an analysis of 1218 casework extractions. Furthermore, in no case was contamination seen for all 4 amplification reactions needed for complete mtDNA haplotyping (Melton and Nelson, 2001).

Thus, the original mtDNA validation studies showed that PCR amplification and subsequent sequencing of PCR products is a reliable method for mtDNA typing from hair, blood and semen. Although significant precautionary methods must be employed to minimize the potential for sample contamination during processing, the approach yields reproducible and dependable results.

Sequence analysis of mtDNA has since then been used to identify weathered skeletal remains for which nuclear DNA analysis had failed (Holland *et*

al., 1993). In these cases, bone samples were pulverized and the DNA was subsequently isolated (Hochmeister *et al.*, 1991) and sequenced for direct comparison to known maternal reference samples. Identification of skeletal remains, through mtDNA typing has since become a mainstay of the Armed Forces DNA Identification Laboratory, giving closure to families of soldiers that have gone missing in action.

§ 1 - 3 The Problem of Mitochondrial DNA Mixtures in Forensics

While mtDNA analysis is usually advantageous in a forensic context (Wilson *et al.*, 1993), mtDNA sequence data can also be compromised by contaminating DNA (*i.e.*, DNA from a minor source contributor) (Wilson *et al.*, 1995; Budowle *et al.*, 2000). As pointed out previously, studies to date suggest that as long as the contaminating DNA accounts for less than 10% of the amplified product it will not compromise the accuracy of sequencing results obtained for the major source amplicon. While this makes sequencing somewhat "tolerant" of low-level DNA contamination, it also prevents the accurate detection and characterization of many cases of sequence heteroplasmy (*i.e.*, those where the heteroplasmic sequence is <10% of the major haplotypes). Conversely, the presence of one or more DNA sequence variants (especially length variants) at levels greater than 10% of the amplified major source product makes interpretation of sequence data difficult if not impossible. The challenge of analyzing mtDNA mixtures is one of the most significant obstacles to the

broader use of mtDNA in forensics. Mixtures can be either "natural" (*i.e.*, heteroplasmy within an individual) or "situational" (*i.e.*, resulting from a mixture of DNA from multiple unrelated individuals). In forensics, natural or situational mixtures of mtDNA molecules that differ by sequence or length can prevent an analyst from being able to determine the true level of discrimination between sequences that are thought to "match" (Fourney, 1998). Variations in DNA sequence result in ambiguous base calls while variations in DNA length result in large stretches of unreadable sequence data (Figure 1.2). In these cases, an analyst can try to employ additional sequencing primers in an effort to obtain readable sequence from amplicons that flank rather than traverse the site of the length variant. Even when this approach works, however, the total amount of sequence data that can be used to characterize a given haplotype is reduced (Holland and Parsons, 1999).

Extensive casework records indicate that a significant proportion of hairs (20.1%) are either heteroplasmic or display a mixed profile (Melton *et al.*, 2005). Moreover, the occurrence of non-heteroplasmic mixed mtDNA profiles appears to increase with the age of a sample and is usually not ameliorated even when following extensive validated cleaning methods (Melton *et al.*, 2005). This, however, is likely to represent only the "tip of the iceberg" since samples which are suspected to contain mixtures are usually not accepted for analysis. Therefore, a reliable means of identifying the individual sequences within a

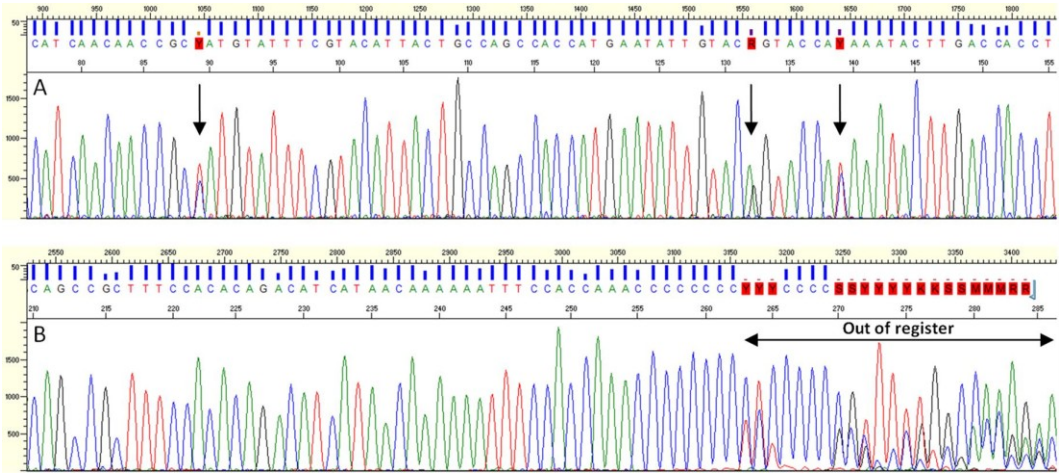


Figure 1.2: DNA Sequencing electropherogram showing ambiguous base calls as indicated by overlapping electrophoretic peaks (arrows in Figure 1.2A) and unreadable sequence data resulting from a mixture of mtDNA length variants (Figure 1.2B). Length variants cause the sequence to fall out of register beginning at the site of the insertion/deletion that produces the variation in length.

mixture could greatly aid investigators by increasing the range of casework samples suitable for mtDNA testing.

§ 1 - 3.1 Current Strategies for mtDNA Mixture Separation

In forensics, medical diagnostics and basic research, the accurate detection of DNA sequence variation is critical. Its excellent resolving power has long made polyacrylamide gel electrophoresis through slab gels or capillaries the technology of choice for DNA sequencing. The mobility and separation efficiency of nucleic acid fragments in these systems, however, can be adversely influenced by the conductivity difference between the analyte and the surrounding buffer unless measures are taken to ensure efficient removal of salts from the sample. Additionally, the denaturing gel systems that are the mainstay of mtDNA typing are often incapable of producing useful data from mixed populations of molecules that either differ in sequence but are identical in length or that are nearly identical in sequence but which contain at least one length polymorphism, (*e.g.*, cases of mtDNA sequence and length heteroplasmy).

Several alternative strategies have, therefore, been proposed to separate DNA mixtures. These include denaturing gradient gel electrophoresis (DGGE) (Hanekamp *et al.*, 1996), single-strand conformational polymorphism (SSCP) (Barros *et al.*, 1997) analysis and sub-cloning into bacterial vectors (Hatsch *et al.*, 2007). These approaches are generally time consuming, necessitate multiple

handling steps, require laborious product purification and are not readily adaptable to automation. These factors have all been obstacles to the implementation of these technologies by forensic laboratories. Both DGGE and SSCP require manual recovery of fractionated DNA from polyacrylamide gels and a second round of PCR amplification to generate enough template for DNA sequencing. This poses a significant problem in a forensic context as reamplification of PCR products increases the risk of contamination and effects of polymerase-mediated nucleotide misincorporation events. Also, in comparative studies, SSCP is only able to detect mixtures in 50-97.5% of samples depending upon the length and base sequence of the analyzed fragment (Gross *et al.*, 1999; Dobson-Stone *et al.*, 2000). Similar results were found for other methods including DGGE (Liu *et al.*, 1998; O'Donovan *et al.*, 1998; Jones *et al.*, 1999).

Subcloning is an even more time and labor-intensive approach. It would require forensic scientists to screen and sequence DNA from multiple transformed bacterial colonies to ensure that observed sequence differences reflect genuine contributors to the starting template rather than artificial variants that were introduced as a result of DNA nucleotide misincorporation by the *Taq* polymerase used for PCR. While alternate methods (*e.g.*, sub-cloning) could readily separate the contributors to a mixture, such approaches are time consuming; contamination prone due to increased sample manipulation and

interpretation of the resulting data can be compromised by artificial sequence variants that frequently arise.

§ 1 - 3.2 The Current State of DHPLC Technology

Denaturing HPLC, widely used in medical research, is an accurate method for rapid, low-cost detection of DNA mixtures and fractionation into their individual components in preparation for subsequent characterization by direct DNA sequencing. While doing so, DHPLC addresses the limitations of the alternative strategies described above. Forensic laboratory implementation of a commercial DHPLC analysis system can be achieved with minimal training at a very reasonable startup cost that is comparable to that of existing DNA sequencing technologies. Seamless incorporation, integrated software and minimal sample consumption also allow for a high degree of automation and streamlining of sample processing and analysis. Finally, the reagent cost for DHPLC analysis is significantly less than that for alternative approaches.

Denaturing HPLC is a method of comparative nucleic acid sequence detection that employs crude PCR products and which is not compromised by the presence of salts or sequence/length heteroplasmy. As originally developed, the technique allows comparative sequence analysis based on the chromatographic separation of denatured and renatured nucleic acids. DHPLC enables the fractionation and subsequent recovery of a DNA mixture on the basis of either

nucleotide sequence or length. Thus, it is possible to isolate and identify the individual contributors to a mixture even in cases where two DNA molecules are identical in length but differ in sequence. The fundamental principle that makes this possible is Temperature Modulated Heteroduplex Analysis (TMHA). This process separates DNA molecules into a series of discernable chromatographic peaks (Huber *et al.*, 1993) (Figure 1.3). The injected DNA binds to a hydrophobic HPLC column in a buffer of triethylammonium acetate (TEAA), which acts as an ion pairing reagent between the negatively-charged backbone of the DNA and the alkylated poly(styrene-divinylbenzene) resin of the HPLC column (Huber *et al.*, 1993). The chemistry and nonporous structure of the column matrix ensure high-resolution nucleic acid separation with rapid regeneration and equilibration between runs.

The DNA is eluted from the column with an increasing gradient of acetonitrile at an elevated temperature. Under these conditions, partial denaturation of double-stranded DNA occurs such that even subtle sequence differences (*i.e.*, single nucleotide substitutions, insertions, deletions) between DNA molecules are readily indicated by the appearance of one or more early eluting peaks/shoulders in the resulting chromatographic profile. These early eluting (*i.e.*, shorter retention time) peaks are produced by heteroduplex

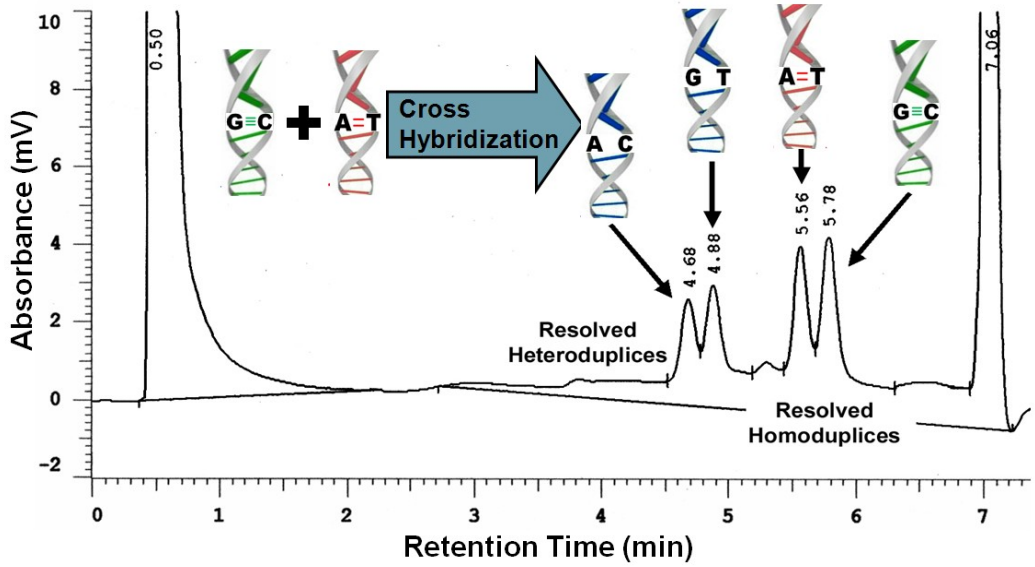


Figure 1.3: DHPLC chromatographic trace of a mtDNA mixture illustrating hetero- and homoduplices produced by cross-hybridization of two HV2A amplification products. One amplicon has an A/T basepair while the other has a G/C basepair at the same site. The earliest eluting peak contains the most destabilizing basepair mismatch (*i.e.*, A/C) while the latest eluting peak has the most stabilizing basepair (*i.e.*, G/C) at the same position.

molecules that denature and elute at lower concentrations of acetonitrile than corresponding homoduplices (O'Donovan *et al.*, 1998; Hou and Zhang, 2000). As illustrated in figure 1.3, the retention times at which individual heteroduplex and homoduplex peaks resolve is a function of the specific base composition, nucleotide mismatch(es) and nearest neighbor interactions of the molecules being separated. Accordingly, the presence of a single chromatographic peak is consistent with sequence identity between the questioned and reference samples. Conversely, the presence of more than one peak is consistent with sequence non-identity between a questioned and reference sample.

A major challenge for DHPLC-based mtDNA mixture fractionation has been the difficulty of completely eliminating the secondary component of a mixture from all recovered fractions. While it is often possible to significantly enrich for one component over another, some fractions still show secondary peaks (*i.e.*, mixed bases) on sequencing electropherograms. Though the electrophoretic peaks for these minor-components are reduced relative to non-fractionated samples, their presence can complicate the interpretation of the mixture. Initial efforts to minimize the presence of these secondary peaks focused on improving the precision with which individual chromatographic peaks are captured. Although this has improved the quality of separations that can be achieved, discussions with Terry Melton (Mitotyping Inc., State College, PA) have resulted in an innovative strategy that allows DHPLC to resolve most if not all

mixtures. Several key experiments (detailed in chapters 5 and 6) have demonstrated the accuracy and reliability of this approach, now coined “linkage phase analysis.”

§ 1 - 3.3 Linkage Phase analysis

Although the term linkage analysis originates from the study aimed at establishing linkage between genes on a chromosome, due to their close proximity and tendency of being inherited together (Ott, 1999), linkage phase analysis in a forensic mtDNA context applies the concept of coordinated nucleotide linkage to the analysis of DNA sequence data from mixed samples.

The underlying principle of linkage phase analysis is that peak heights on sequencing electropherograms reflect the relative efficiency with which DNA molecules are terminated at a given position together with the efficiency of laser-induced florescence of individual dideoxy dye-terminators. While sequence context results in significant peak height differences among individual bases, these relative differences in the height of any one peak relative to any other peak are highly reproducible. When a DNA mixture is sequenced, overlapping electrophoretic peaks occur wherever the amplicons differ in primary sequence. Because peak height is sequence context dependent, measurements of peak height alone cannot be used to determine the relative abundance of the amplicons within the mixture. However, studies have shown that a change in the

ratio of one component to another in a mixture produces a corresponding change in the ratio of the fluorescence signals for the overlapping electropherogram peaks (Danielson *et al.*, 2007). In other words, the peak heights for all bases associated with one amplicon will increase while the peak heights for all bases associated with the other amplicon will decrease. By analyzing this "linkage phase" of the bases at all mixed base positions, the correct haplotype for each contributor to a mixture can be reliably determined.

§ 1 - 3.4 Impact on the Criminal Justice System

The practical significance of DHPLC-based mtDNA mixture fractionation is that this technology may aide criminal investigations by making it possible to obtain crucial probative information in cases where existing methods yield ambiguous or uninterpretable results. The successful forensic validation of DHPLC for mtDNA mixture analysis will provide the forensic community with tools to efficiently fractionate and interpret mixtures of mtDNA in casework samples. As an added benefit, DHPLC has the potential to streamline the overall process of mtDNA analysis. This has the potential to provide the analyst with a rapid and cost-effective means of assessing mtDNA sample integrity and determining whether a sample is a mixture requiring fractionation. Once such a determination has been made, DHPLC yields a product which is ready for conventional sequencing analysis without reamplification or further purification.

The equipment start up cost for DHPLC is comparable to other analytical instruments used in forensic DNA laboratories such that implementation of DHPLC-based analysis can be achieved with minimal training and at a reasonable equipment startup cost ($135K). The cost of separating and recovering individual components of a DNA mixture by DHPLC is significantly less than it would be for the far more labor-intensive electrophoretic techniques or subcloning. Separation and recovery of a two-contributor mixture of DNA by DHPLC can be performed for less than $10 in reagent cost. The technology also offers analysts a significant opportunity to streamline the standard workflow at nearly every step in the process. On the front side, DHPLC makes it possible to determine with near 100% accuracy whether an amplified mtDNA sample consists of a single amplicon or represents a mixed product (*i.e.*, either a heteroplasmic or situational mixture). If the product is mixed, it is often possible to determine by DHPLC the approximate level of complexity (*e.g.*, 2 contributors vs. >2 contributors). Also on the front end of the mtDNA amplification process, DHPLC makes it possible to very accurately determine the amount of template amplified in the PCR process and to simultaneously purify the target template for optimal dye terminator labeling. This not only eliminates the need for laborious yield gels and expensive PCR product clean up columns but also ensures that the analyst is using an optimal quantity and quality of mtDNA for the dye terminator labeling reaction. In the case of mixed samples, fractionation allows the mtDNA sequence of

individual contributors to be unambiguously determined without secondary amplification or excessive manipulation. Finally, this technology is amenable to a high degree of automation with minimal sample consumption. *In toto*, the ability to accurately analyze evidentiary material that is not amenable to existing methods of analysis while also enabling the forensic analyst to streamline the overall analytical process would represent a significant contribution to enhanced justice and public safety.

§ 1 - 4 Objectives and Soundness of the Dissertation Research

A mixture of different mtDNA molecules in a single sample is a significant obstacle to the successful use of standard methods of mtDNA analysis (*i.e.,* dideoxy dye-terminator sequencing). Forensic analysts often encounter either naturally occurring mixtures (*e.g.*, heteroplasmy) or situational mixtures typically arising from a combination of body fluids from separate individuals. The ability to accurately resolve and interpret these types of samples in a timely and cost efficient manner would substantially increase the power of mtDNA analysis and potentially provide valuable investigative information by allowing its use in cases where the current approach is limited or fails. Therefore, the ultimate goal of this research is to develop a strategy for the use of DHPLC as a developmentally-validated forensic application for resolving mixtures of mtDNA. To facilitate the adoption of this technology by the forensic community, a significant effort has been made to ensure that this technology meets SWGDAM developmental

validation criteria and interfaces smoothly with previously validated methods of forensic mtDNA analysis. To do this, the method developed using DHPLC employs mtDNA amplicons, PCR conditions and DNA sequencing protocols validated for use in forensic laboratories.

§ 1 - 4.1 Hypothesis

The central concept being tested is that DHPLC will provide a rapid, accurate, and cost effective method of separating DNA mixtures. This will increase the number of forensic samples for which definitive sequence analyses will be possible. The specific experimental core hypotheses of this project are that:

(1) DHPLC will be able to sufficiently detect and fractionate mtDNA mixtures regardless of the location (*i.e.,* base position) and number of mixed sites being assayed.

(2) There will be a significant correlation between a change in DNA mixture ratios (*i.e.,* based on quantity) and DNA electrophoretic peak height ratios at all mixed base positions and that this will allow for high-confidence linkage phase analysis of mtDNA mixtures for the subsequent determination of individual contributor haplotypes.

(3) DHPLC fractionation and linkage phase analysis will have the quantitative sensitivity, reproducibility and accuracy to meet forensic / SWGDAM validation standards.

§ 1 - 4.2 Summary of Research Goals

§ 1 - 4.2.1 Chapter 2

The focus of Chapter 2 are experiments to support the development of a single specific DHPLC assay for each of the four forensically relevant fragments of the D-loop of mtDNA for the purpose of detecting sequence identity/non-identity. This will serve as a presumptive test for detection of mixed mtDNA amplicons arising from a two (or more) contributor mixture.

§ 1 - 4.2.2 Chapter 3

The focus of Chapter 3 is a set of experiments to determine the applicability of HPLC for the purification and accurate quantification of PCR amplicons prior to dye-terminator labeling. The combination of the purification and quantification process into a single step will minimize sample consumption, decrease analysis time and provide an opportunity for automation.

§ 1 - 4.2.3 Chapter 4

The focus of Chapter 4 is a set of experiments to investigate the degree of consistency in context-specific differences in relative peak heights across mtDNA sequencing electropherograms. Additional tests evaluate the use of a DNA

sequence electropherogram pattern matching algorithm for the detection of peak height anomalies, changes in peak height context specificity and mutation analysis by comparison to a reference sequence pattern. This computational approach will facilitate the automated analysis of sequence electropherograms allowing the analyst to more readily focus on genuinely anomalous areas of sequence and to, therefore, interpret results with greater confidence.

§ 1 - 4.2.4 Chapter 5

The focus of Chapter 5 is a set of experiments designed to determine the extent to which mtDNA mixtures can be analyzed as a function of changes in overlapping electrophoretic peak height fluorescence ratios at individual mixed base positions. A strong correlation between a change in DNA quantity ratio and a corresponding change in DNA peak height ratio will allow for the accurate determination of relative DNA quantities and thus linkage phase for virtually all two-contributor mixtures and the subsequent ability to correctly identify the individual mtDNA haplotypes present in a mixture.

§ 1 - 4.2.5 Chapter 6

The focus of Chapter 6 is a set of experiments designed to determine the efficacy of DHPLC for the fractionation of two-component mtDNA mixtures. The efficacy with which sequence-ready DNA can be recovered without reamplification represents a critical bench mark. The development of a statistical

approach for the accurate determination of linkage phase of the individual contributors to a mtDNA mixture, will also be presented.

§ 1 - 4.2.6 Chapter 7

The focus of Chapter 7 is a series of studies designed to validate the linkage phase approach, detailed in Chapter 6. Using simulated forensic samples, DHPLC in combination with linkage phase analysis will be used to predict the haplotypes of contributors to a series of two-component mixtures. The results of these studies will establish the applicability and reliability of statistical approaches to the analysis of sequence data from DHPLC-fractionated mixtures.

§ 1 - 4.2.7 Chapter 8

The focus of Chapter 8 is a series of studies designed to validate the use of the DHPLC instrumentation for mtDNA analysis of forensic casework samples. These include tests for column and injection reproducibility, column and fraction-to-fraction cross-contamination, detection sensitivity, concentration accuracy and determination and negative control screening. The results of these studies will serve to validate DHPLC in accordance with SWGDAM and DNA Advisory Board (DAB) standards.

§ 1 - 4.2.8 Chapter 9

The focus of Chapter 9 is a summarization of an optimized overall sample process flow for mtDNA analysis by DHPLC, prior to dye-terminator sequencing.

Additionally, data are presented to demonstrate and validate the mixture detection and linkage phase analysis capabilities of a prototype expert bioinformatic system, *i.e.,* the Fractional Linkage Phase Analysis Resource Software (FLiPARS).

Chapter 2: Comparative Sequence Analysis of the HV1 and HV2 Regions of Human Mitochondrial DNA by Denaturing High-Performance Liquid Chromatography

§ 2 - 1 Introduction

Analysis of mtDNA for forensic casework is relatively expensive, time consuming and requires the expertise of skilled analysts currently employed by a limited number of public and private laboratories. Many efforts to streamline the processing of forensic mtDNA casework have sought to develop rapid, cost effective and reliable methods of comparative mtDNA sequence analysis to "assay" for sequence identity/non-identity between samples or for the presence of mtDNA mixtures within a single sample prior to direct sequence characterization.

A variety of approaches for mtDNA comparative sequence analysis have been investigated in recent years. These include, hybridization to linear arrays of sequence-specific oligonucleotides (SSO) (Reynolds *et al.,* 2000; Gabriel *et al.,* 2001); denaturing gradient gel electrophoresis (DGGE) (Hanekamp *et al.,* 1996; Steighner *et al.,* 1999); single-strand conformational polymorphism (SSCP) analysis (Alonso *et al.,* 1996; Barros *et al.,* 1997); time-of-flight mass spectrometry (Butler and Becker 2001); and microarray-based analysis

(Fukushima 1999). These approaches, suffer from a number of limitations which include: the interrogation of only a subset of variant sites; cross-hybridization to non-target sequences; laborious manipulations of gels (Meyers *et al.,* 1988; Reynolds *et al.,* 2000); and interference by primer impurities (Butler and Becker 2001). Most critically, however, all of these approaches suffer from the fact that they consume often precious forensic evidence while not necessarily providing a reliably comprehensive assessment of all possible sequence differences across the entire amplicon.

Denaturing high-performance liquid chromatography (DHPLC) circumvents these limitations while offering the potential to characterize sequence differences between mtDNA amplicons in an accurate, rapid and cost-effective manner that can be automated. Moreover, DHPLC makes it possible to recover the assayed DNA from the column eluent at the end of an assay. As currently employed in a variety of biomedical applications, the technique facilitates discrimination between amplicons that differ in sequence (*i.e.*, single nucleotide substitutions, insertions, deletions) but which may be identical in length. When all DNA molecules in a sample are identical, denaturation and renaturation produce only homoduplices which elute simultaneously to yield a single chromatographic peak in a DHPLC assay. Conversely, a mixture of non-identical DNA amplicons yields a combination of homo- and cross-hybridized heteroduplices. Heteroduplices elute more readily than homoduplices

(O'Donovan *et al.,* 1998; Hou and Zhang 2000) from the DHPLC DNASep® column. Thus, sequence non-identity between DNA molecules is readily indicated by the appearance of one or more early-eluting heteroduplex peak(s) on the resulting chromatogram (Huber *et al.,* 1993; O'Donovan *et al.,* 1998; Hou and Zhang 2000).

In comparative studies of DHPLC-based detection of sequence polymorphisms versus alternate approaches such as SSCP and DGGE, DHPLC has often been found to provide superior sensitivity and accuracy (Abrams *et al.,* 1990; O'Donovan *et al.,* 1998; Gross *et al.,* 1999). Using carefully designed assay conditions, the concordance between direct DNA sequencing and comparative sequence analysis by DHPLC typically ranges from 95-100%. In some cases, DHPLC has proven to have greater sensitivity than direct DNA sequencing for the detection of minor component sequence variants. The accuracy and sensitivity with which sequence variants can be detected by DHPLC can be significantly influenced by the length, GC content and other thermodynamic characteristics of the amplicon being assayed. The current study, therefore, investigated the potential utility of DHPLC to specifically detect sequence variants throughout the forensically relevant portions of the HV1 and HV2 regions of human mtDNA. A high degree of concordance between DHPLC and direct sequencing for the comparative analysis of those amplicons commonly used by forensic practitioners could facilitate the development of less consumptive approaches to comparative mtDNA sequence analysis for screening purposes.

§ 2 - 2 Methods

This research was conducted in compliance with U.S. Federal Policy for the Protection of Human Subjects (56 FR 28003). Blood samples were collected from 103 unrelated subjects who had provided informed consent, of these, 8 samples were used to generate of mtDNA subclones. All samples were stored at -20°C until DNA extraction.

§ 2 - 2.1 Mitochondrial DNA Extraction from Dried Blood

Total DNA was extracted from dried reference blood stains using a QIAmp® 96 Well Blood Kit (Qiagen Inc., Valencia, CA) per the manufacturer's recommended protocol. For consistency, the extraction was performed using a Biomek® 2000 automated liquid handler (Beckman Coulter Inc, Fullerton, CA). Purified DNA was eluted into 200µL of nuclease free ddH_2O and stored at -20°C until PCR amplification.

§ 2 - 2.2 Mitochondrial DNA Amplification and Sequencing

Mitochondrial DNA control region amplification reactions (50µL) were prepared using 50pmol each of forensically-validated PCR primers (Wilson *et al.,* 1995; LaBerge *et al.,* 2003) (Table 2.1); 2.25U Ampli*Taq* GOLD® DNA polymerase (Applied Biosystems, Foster City, CA) supplemented with 0.25U *Pfu* DNA polymerase (Stratagene, La Jolla, CA); Ampli*Taq* GOLD® Buffer (Applied Biosystems); 10nmol of each dNTP (Stratagene) and 10µL of an approximately

Table 2.1: Forensically validated and additional primers developed in-house for control region amplification. Location indicates strand corresponding to the primer (L for light strand and H for heavy strand) followed by the nucleotide position of the 3’ end of the primer.

Primer	Location	Sequence
A1	(L15997)	5’ CAC CAT TAG CAC CCA AAG CT 3’
A2	(L16159)	5’ TAC TTG ACC ACC TGT AGT AC 3’
B1	(H16391)	5’ GAG GAT GGT GGT CAA GGG AC 3’
B2	(H16236)	5’ CTT TGG AGT TGC AGT TGA TG 3’
C1	(L48)	5’ CTC ACG GGA GCT CTC CAT GC 3’
C2	(L172)	5’ ATT ATT TAT CGC ACC TAC GT 3’
D1	(H408)	5’ CTG TTA AAA GTG CAT ACC GCC A 3’
D2	(H285)	5’ GGG GTT TGG TGG AAA TTT TTT G 3’
D2-3'G	(H286)	5’ GGG GTT TGG TGG AAA TTT TTT 3’
HV1R	(H16485)	5' GGA ACC AGA TGT CGG ATA CAG TTC 3'
HV2F	(L16498)	5' CAT CTG GTT CCT ACT TCA GGG TCA 3'

10pg/µL DNA extract. Amplifications were performed on a GeneAmp® 9700 thermocycler (Applied Biosystems) with an initial denaturation at 95°C for 10 minutes, followed by 32 cycles of 95°C for 20 seconds, 60°C for 30 seconds and 72°C for 45 seconds. The final extension at 72°C was for 15 minutes. The resulting PCR yield for each fragment was then quantified by HPLC under a non-denaturing column temperature of 50°C. The near perfect regression (R^2 = 0.9982) between chromatographic peak area and DNA quantity made it possible to reliably determine the DNA quantity from each amplification reaction. This approach, which requires ≤7 min/assay, has been internally validated for fragments ranging in size from 200-1000bp and for quantities ranging from 1.25-140ng (data not shown).

Approximately 0.8ng of amplified DNA was labeled for sequencing using the BigDye® Terminator v1.1 Cycle Sequencing kit (Applied Biosystems). Labeling reactions were primed using the PCR primers employed above or additional internal sequencing primers (HV1R and HV2F) developed in-house (Table 2.1). Labeled products were purified by ethanol precipitation or on Performa® DTR V3 96-Well Short Plates (Edge BioSystems, Gaithersburg, MD) and then resolved on a PRISM® 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocols using the POP-6™ polymer and 47cm x 50µm capillaries (Applied Biosystems). The raw electrophoretic traces were analyzed using the KB Basecaller (Applied Biosystems) together with the appropriate dye set mobility

file for the v1.1 kit. Appropriate positive and negative controls were included at all stages of the process to monitor for contamination and reagent integrity. The resulting sequence data were analyzed using the Sequencher™ v4.2 DNA analysis software (Gene Codes Corp, Ann Arbor, MI).

§ 2 - 2.3 Mitochondrial DNA Subcloning

Primers A1 and D1 (Table 2.1) were used to amplify an mtDNA fragment of approximately 1021bp which encompassed both HV1 and HV2. The resulting amplification products representing eight mtDNA haplotypes were purified using Wizard® PCR Preps (Promega, Madison, WI), ligated into the pGEM®-T vector (Promega) and electroporated into DH5α electrocompetent cells. Cells were selected on LB agar plates containing 50μg/ml ampicillin and screened by PCR to identify plasmids containing target inserts. Plasmids were isolated from overnight cultures of these colonies by alkaline lysis and capture on glass-filter binding plates (Millipore, Bedford, MA). DNA was sequenced as described above.

§ 2 - 2.4 DHPLC-Based Temperature-Modulated Heteroduplex Analysis (TMHA)

DNA sequence data for each of four forensically-relevant amplicons (HV1A, 278bp: A1/B2; HV1B, 271bp: B1/A2; HV2A, 278bp: C1/D2; and HV2B, 277bp: D1/C2) (Table 2.1) were compared to identify individuals with different mtDNA haplotypes (Wilson *et al.,* 1995). Fragments were then combined at a 1:1 molar ratio to generate a series of pair-wise mixtures which were cross-

hybridized by denaturation at 95°C for 4 minutes and gradual cooling (1.5°C/min) to a final temperature of 25°C (LaBerge *et al.,* 2003).

The cross-hybridized mixtures were analyzed by TMHA (Kuklin *et al.,* 1997) on a WAVE® 3500HT DNA Fragment Analysis System (Transgenomic Inc., Omaha, NE) using a DNASep® analytical column (Huber *et al.,* 1993). Triethylammonium acetate (TEAA) pH 7.0 at a final concentration of 0.1M served as an ion-pairing reagent. Cross-hybridized mixtures were analyzed under partially denaturing conditions at the empirically determined optimal temperatures for each of the four mtDNA amplicons (*i.e.*, HV1A, 58°C; HV1B, 59.2°C; HV2A, 56.5°C and HV2B, 57°C). The optimal acetonitrile linear gradient generated from differential mixing of buffer A (0.1M TEAA) and buffer B (0.1M TEAA, 25% ACN) were a 56% to 65% buffer B increase in 3.5 minutes for HV1A, HV2A and HV2B and a 55% to 64% buffer B increase in 3.5 minutes for HV1B. All samples were eluted at a 0.9ml/min flow rate and detected by UV absorbance at 260nm. Standard DHPLC controls included: zero-volume injections to screen for DNA carryover between assays; no-template PCR controls to check for reagent contamination; and manufacturer mutation control standards for buffer and column quality control. Where necessary for enhanced sensitivity, temperate titrations were conducted in ±0.5°C increments using an integrated oven accurate to ±0.1°C.

The resulting chromatograms were visually examined. The presence of a single homoduplex peak was scored as consistent with sequence identity. The presence of more than one chromatographic peak or a shoulder on a homoduplex peak was scored as consistent with the presence of one or more sequence differences between the two amplicons in the sample being assayed, *i.e.*, sequence non-identity (O'Donovan *et al.,* 1998; Hou and Zhang 2000).

§ 2 - 3 Results and Discussion

§ 2 - 3.1 Essential Modification of Forensically-Validated Protocols

In evaluating the utility of DHPLC for comparative mtDNA sequence analysis, the current study employed forensically validated oligonucleotide primer pairs and standard operating procedures (Wilson *et al.,* 1993). Two minor modifications to these procedures were made, the exclusion of bovine serum albumin (BSA) from the amplification reaction and the supplementation of Ampli*Taq* GOLD® with higher fidelity *Pfu* DNA polymerase.

The presence of >10% w/v BSA in a sample reduces the functional lifespan of the DNASep® column. BSA, however, is often used to ameliorate the effects of endogenous PCR inhibitors in some DNA extracts. These include IgG and heme in blood (Al-Soud and Rådström 2001), melanin in hair (Eckhart *et al.,* 2000), collagen in bone (Scholz *et al.,* 1998) and such environmental inhibitors as humic acid in soil (Watson and Blackwell 2000) which are not efficiently removed

by inorganic extraction methodologies (*e.g.*, Chelex™). In our laboratory, both endogenous and environmental inhibitors have been effectively removed from hair, bone and environmentally contaminated samples by the use of organic extraction, silica-gel membranes or by systems that employ paramagnetic particles such as the Qiagen® EZ1 DNA tissue kit.

In the current study, the silica-gel membrane based QIAamp® DNA Blood purification kit was used to prepare all DNA extracts from whole blood. The purity of the resulting extracts was evaluated by PCR amplification of mtDNA in the presence and absence of BSA at a final concentration of 0.16µg/µl. Yields for individual amplification reactions were generally in the range of 20-25ng/µl as determined by optical absorbance at 260nm. The amplification of mtDNA from QIAamp® prepared extracts was equally efficient regardless of whether or not BSA was added (data not shown). If the use of BSA is unavoidable, purification of the PCR amplification products by organic extraction or other protein removal method may be needed prior to DHPLC analysis.

The fidelity of the DNA polymerase used for amplification may also impact the accurate interpretation of DHPLC chromatograms. The relatively high nucleotide misincorporation rate of *Taq* DNA polymerase (8.0×10^{-6} mutations/bp/duplication) (Cline *et al.,* 1996) typically produce DHPLC chromatograms from single sequence samples which erroneously appear to

indicate a DNA mixture. A mixture of *Taq* and *Pfu* DNA polymerases at a 9:1 ratio effectively eliminates these anomalies. The 3′→5′ exonuclease activity and lower nucleotide misincorporation rate (1.3×10^{-6} mutations/bp/duplication) of *Pfu* yields greater uniformity in amplification products (Cline *et al.,* 1996).

§ 2 - 3.2 DHPLC Assay Development

Recognizing that mtDNA heteroplasmy is detected by DHPLC as a mixture of non-identical sequences, the initial optimization of DHPLC assay parameters was conducted using a group of eight mtDNA subclones as templates for PCR amplification of the HV1 and HV2 amplicons (Table 2.2). Collectively, these subcloned sequences differed from each other at an average of 9.4 positions (0-7 positions in HV1A; 0-10 positions in HV1B; 0-5 positions in HV2A; 0-4 positions in HV2B). The use of subcloned sequences ensured that each sample represented a single distinct haplotype. This eliminated heteroplasmy as a potentially confounding variable for the purpose of assay optimization.

§ 2 - 3.3 Optimization of DNASep® Column Temperatures

Comparative DHPLC-based DNA sequence analysis relies on temperature-dependent chromatographic separation of the nucleic acids under partially denaturing conditions. In accordance with manufacturer recommendations and empirical observations by the authors and other researchers, optimal resolution is generally obtained using amplicons of less than 400bp in length and a column

Table 2.2A: Sequence polymorphisms in HV1 subclones based on the revised Cambridge Reference Sequence (rCRS).

HV1	Nucleotide Position (rCRS)																			
	HV1A							HV1B												
Sample	16086	16104	16224	16126	16128	16129	16146	16182	16183	16189	16193.1	16223	16249	16294	16296	16304	16311	16323	16342	16359	16362
rCRS	**T**	**C**	**T**	**T**	**C**	**G**	**A**	**A**	**A**	**T**	**:**	**C**	**T**	**C**	**C**	**T**	**T**	**T**	**T**	**T**	**T**
Subclone 1	*	*	*	*	*	A	*	*	C	C	C	T	C	*	*	*	C	*	*	C	*
Subclone 2	*	*	*	C	*	*	*	*	*	*	*	*	*	T	T	C	*	*	*	*	*
Subclone 3	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Subclone 4	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Subclone 5	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C
Subclone 6	*	T	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	C	*	*
Subclone 7	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*
Subclone 8	*	*	*	*	*	*	*	C	C	C	*	*	C	*	*	*	*	*	*	*	*

Table 2.2B: Sequence polymorphisms in HV2 subclones based on the revised Cambridge Reference Sequence (rCRS).

HV2	Nucleotide Position (rCRS)												
	HV2A						HV2B						
Sample	57.1	64	73	146	152	195	263	282	285	309.1	310	315.1	385
rCRS	**:**	**C**	**A**	**T**	**T**	**T**	**A**	**T**	**C**	**:**	**T**	**:**	**A**
Subclone 1	*	*	G	*	*	C	G	*	*	C	*	C	*
Subclone 2	*	*	G	*	*	*	G	*	*	C	*	C	*
Subclone 3	*	*	*	*	*	*	G	*	*	*	*	C	*
Subclone 4	*	*	*	*	C	*	G	*	*	*	*	C	*
Subclone 5	C	T	*	*	*	*	G	*	*	*	*	C	*
Subclone 6	*	*	G	*	*	*	G	C	*	C	*	C	*
Subclone 7	*	*	G	C	C	*	G	*	*	C	C	C	*
Subclone 8	*	*	G	*	*	*	G	*	T	*	*	C	G

Due to a 285C>T mutation in the native sequence from which Subclone 8 was derived, a 1nt truncated D2 primer was used for all amplification reactions.

temperature where the average of nucleic acid helicity across an amplicon is approximately 75% to 85% (Cooksey *et al.,* 2002; Transgenomic 2003). Under these conditions, heteroduplices with a single base mismatch elute from the DNASep® column before identical-length homoduplices.

The mtDNA primer pairs used in the current study yield amplicons within a size range that are well suited for DHPLC analysis (*i.e.*, HV1A = 278bp, HV1B = 271bp, HV2A = 278bp, HV2B = 277bp). Although the appropriate partially denaturing temperature for a given amplicon is approximated by the Navigator™ software based on nearest neighbor interactions (Fixman and Freire 1977), the optimal temperature must be empirically confirmed. This was done by monitoring the separation of homo- and heteroduplices in multiple mixed samples at temperatures flanking the predicted value. The optimal temperature is that at which the greatest chromatographic resolution is achieved. This is illustrated in figure 2.1 where the greatest resolution for the mixture of HV1B amplicons being assayed is at 59.2°C. This is not to suggest that all mixtures of HV1B amplicons will be optimally resolved at this temperature. Rather, this represents the optimal initial assay temperature at which mixtures of HV1B amplicons of unknown haplotype can be screened. Similarly, the optimal initial assay temperatures for the HV1A, HV2A, and HV2B amplicons were determined to be 58.0°C, 56.5°C and 57.0°C, respectively.

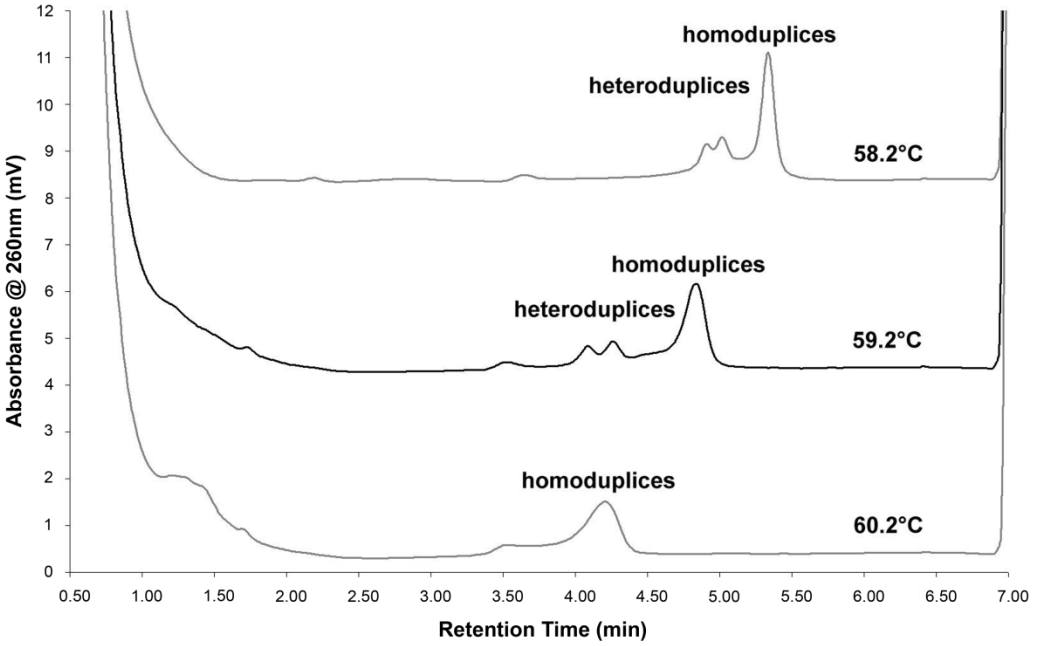


Figure 2.1: Empirical determination of the optimal column temperature for comparative sequence analysis of HV1B. While the hetero- and homoduplices can be resolved at 58.2°C, optimal resolution is obtained at 59.2°C. At 60.2°C, the heteroduplices elute too readily to form distinct peaks.

§ 2 - 3.4 Comparative Sequence Analysis of Subcloned mtDNA

As a preliminary assessment of the accuracy with which DHPLC is able to detect sequence differences, a matrix of all possible pair-wise combinations (*i.e.*, 144 mixtures) of mtDNA fragments (*i.e.*, HV1A, HV1B, HV2A and HV2B) was prepared using the subcloned haplotypes as amplification templates. Following cross-hybridization and analysis by DHPLC, the resulting chromatograms were visually examined.

Figure 2.2 presents examples of chromatograms obtained from assays of 27 pair-wise comparisons between non-identical and 9 comparisons between identical HV2A amplicons. Sequence differences included a single-base insertion situated near the terminus of the amplicon (*e.g.*, all comparisons with sample 5) and a variety of single and multiple base substitutions throughout the amplicon. Single base substitutions in both GC rich and AT rich regions were also assayed.

Combinations of each amplicon with itself, and the identical amplicons from subclones 2 and 8, yielded chromatograms with a single symmetrical peak (*i.e.*, the homoduplex species). For two of the pair-wise combinations, non-identity was indicated by the presence of a shoulder on the homoduplex peak (*e.g.*, subclones 2 and 4). For 24 of 27 pair-wise combinations of non-identical amplicons, sequence non-identity was indicated by the presence of early-eluting and easily discernable heteroduplex peaks. In a few comparisons, the heteroduplex peak was much smaller than the homoduplex peak (*e.g.*, subclones

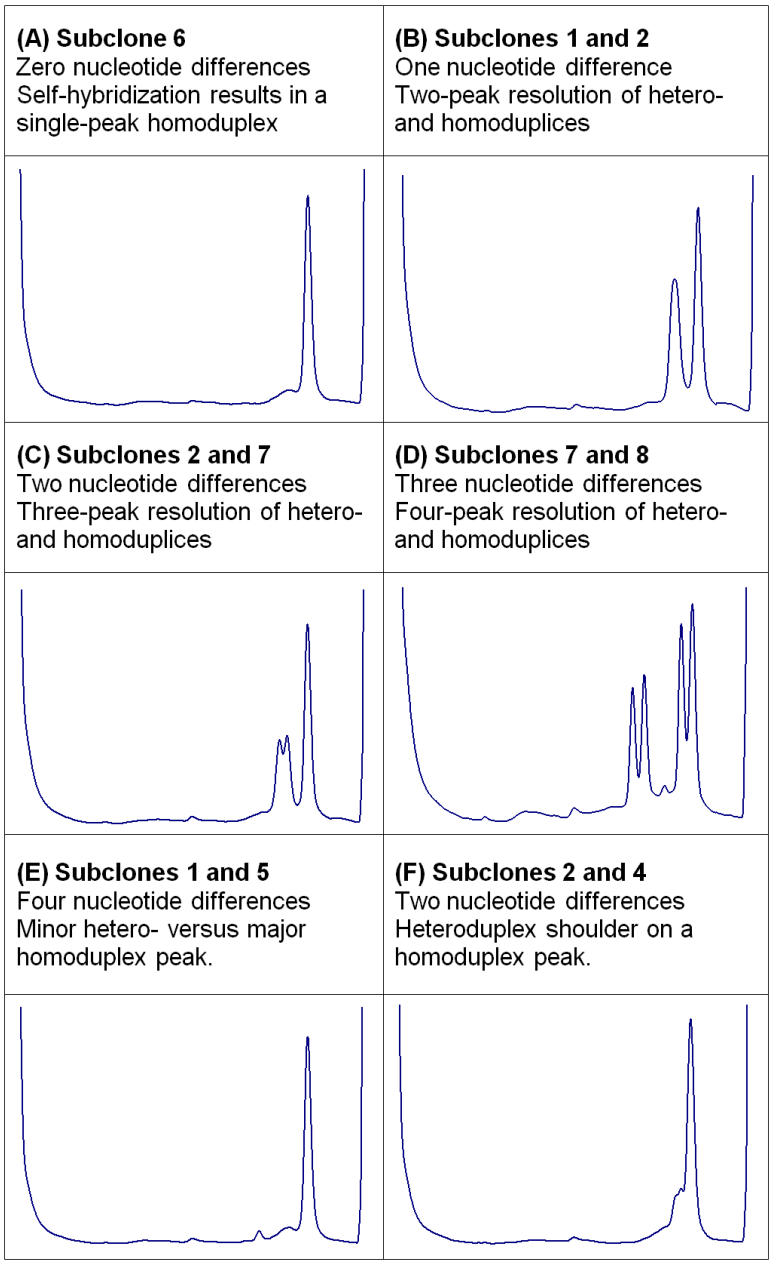


Figure 2.2: Examples of DHPLC chromatograms from pair-wise hybridization of the HV2A fragment amplified from eight mtDNA subclones. (2A) Sequence identity is indicated by the presence of a single homoduplex peak. (2B-2E) Sequence non-identity is indicated by the presence of more than a single peak. Early eluting peaks represent heteroduplices formed by cross-hybridization between non-identical amplicons. (2F) Sequence non-identity can also be indicated by a shoulder on a homoduplex peak which also indicates the presence of heteroduplices.

1 and 5) making detection somewhat more challenging. In these cases, the presence of a heteroduplex peak was more readily discerned by comparing the chromatograms obtained for injections of each amplicon by itself with the chromatogram obtained when a mixture of the two amplicons was injected. This was done by superimposing these three chromatograms on top of each other.

Only the comparison between subclones 2 and 3 failed to show sequence non-identity by DHPLC at 56.5°C. This was not unexpected since the sequence difference between these amplicons (73G vs. 73A) was immediately adjacent to a short GC-rich region of predicted 100% helicity. The thermodynamic stability of this region is an intrinsic feature of the HV2A amplicon which interferes with the detection of base changes at position 73.

Pair-wise combinations of HV1A, HV1B and HV2B fragments amplified from the eight mtDNA haplotype subclones were also assayed for sequence identity/non-identity by DHPLC. As seen for the HV2A fragment, sequences that differed by one or more bases were successfully identified by DHPLC either by the presence of early-eluting heteroduplex peaks or an early-eluting heteroduplex shoulder on a homoduplex peak. One sequence difference between subclones 3 and 5 (16362T vs. 16362C) was not readily detected without overlaying the chromatograms for the individual versus the mixed amplicons. This was not unexpected given that HV1B has a biphasic melt profile

and position 16362 is within a region of higher GC-content that remains relatively stable at the standard assay temperature of 59.2°C.

§ 2 - 3.5 Comparative Sequence Analysis of mtDNA from Blood Samples

The primary objective of the current study was to evaluate the potential utility of DHPLC as a means of screening amplified human mtDNA samples for sequence identity/non-identity in the context of a forensic laboratory. Initial experiments using subcloned mtDNA haplotypes demonstrated that DHPLC accurately detects even single base differences between two mtDNA amplicons. Subclones, however, are artificially pure templates which do not reflect the molecular complexity of a human tissue-derived DNA extract. Furthermore, the eight haplotypes used encompass only a small fraction of known variant positions. To more thoroughly assess the reliability of DHPLC as a tool for comparative sequence analysis, the approach was tested on blood samples from 95 research volunteers.

Exclusive of length polymorphisms associated with the homopolymeric cytosine-stretch in HV2, the 95 subjects in the current study represented 83 distinct haplotypes (Appendix A). Seventy-seven of these were unique within the study population. Of the 6 haplotypes which occurred more than once, the most common (263G, 315.1C) was observed 8 times while the remaining 5 haplotypes each occurred twice. Relative to the revised Cambridge Reference Sequence

(rCRS) (Anderson *et al.,* 1981; Andrews *et al.,* 1999), the haplotypes represented in the current study encompass 84 polymorphisms in HV1 and 46 polymorphisms in HV2, including cytosine-stretch length polymorphisms.

Based on pair-wise comparisons, individual haplotypes differed from each other at 0-22 positions (0-11 in HV1A; 0-13 in HV1B; 0-13 in HV2A; and 0-12 in HV2B). On average, there were 8.71 positional differences between haplotypes. *In toto*, the study population encompasses a broad diversity of haplotypes and thus is well suited for evaluating the utility of DHPLC for the accurate detection of sequence polymorphisms throughout the mtDNA control region. This is essential for the validation of DHPLC as a tool for comparative sequence analysis.

A total of 920 pair-wise combinations of amplicons from the 95 individuals in this study were prepared, denatured and allowed to gradually reanneal. Of these, 72 (22 in HV1A, 8 in HV1B, 17 in HV2A, and 25 in HV2B) represented combinations of amplicons that were from different individuals but which had identical DNA sequences. DHPLC analyses of these samples all produced clear chromatograms consisting of a single symmetrical homoduplex peak. This pattern is consistent with sequence identity and is 100% concordant with direct sequencing data for these amplicons.

To assess the reliability of DHPLC to detect sequence non-identity, 849 combinations of amplicons (209 in HV1A, 222 in HV1B, 213 in HV2A, and 205 in

HV2B) which differed in sequence were assayed. These differences encompassed a broad diversity of polymorphisms distributed throughout the HV1 and HV2 regions including transitions, transversions, insertions and deletions (Table 2.3). Positional differences were located internally as well as near the termini of amplicons and encompassed regions of varying GC richness. In all, the mixtures assayed in this study included sequence variants in HV1A, HV1B, HV2A and HV2B at 39, 62, 38 and 30 different positions, respectively. Figure 2.3 illustrates the distribution of the variant positions and the frequency with which they were assayed.

Using the aforementioned initial assay temperature for each mtDNA amplicon, DHPLC analyses correctly indicated the presence of a mixture of non-identical amplicons in 836 (209 in HV1A, 222 in HV1B, 203 in HV2A, and 202 in HV2B) of the combinations tested. The remaining 13 mixtures (10 in HV2A and 3 in HV2B) of non-identical amplicons, yielded chromatographic traces with a single eluent peak, a result erroneously suggesting sequence identity. Careful examination of the amplicon mixtures which were not detected by DHPLC reveals that these aberrant results are limited to a very small number of challenging positions. Mirroring results obtained with subcloned mtDNA, it was not possible to detect as non-identical, combinations of HV2A amplicons that differ only at positions 72 or 73. Taken together, these two positions account for all of the undetected non-identical mixtures in HV2A.

Table 2.3: Sequence polymorphisms assayed by DHPLC in the HV1 and HV2 regions as deviations from rCRS.

	HV1										HV2					
Transitions	16041G	16051G	16069T	16093C	16104T	16111T	16114T	16124C	16126C	16129A	55C	64T	72C	73G	89C	92A
	16136C	16144C	16145A	16147T	16148T	16153A	16154C	16163G	16172C	16173T	93G	114T	131C	143A	146C	150T
	16176T	16179T	16186T	16187T	16189C	16192T	16193T	16209C	16215G	16217C	152C	153G	185A	185A	188G	189G
	16221T	16222T	16223T	16224C	16239T	16241G	16243C	16244A	16249C	16256T	192C	194T	195C	196C	198T	199C
	16258G	16261T	16266T	16270T	16274A	16278T	16286T	16287T	16290T	16291T	200G	204C	207A	217C	222T	225A
	16293G	16294T	16295T	16296T	16298C	16299G	16301T	16304C	16309G	16311C	226C	228A	234G	239C	242T	250C
	16316G	16320T	16325C	16327T	16343G	16352C	16353T	16354T	16355T	16357C	285T	293C	295T	319C	340T	
	16359C	16362C	16390A													
Transversions	16111A	16129C	16176A	16182C	16183C	16184A	16265C	16290G			57G	207C	280G			
Insertions	16192.1T 16193.1C										309.1C 309.2C					
Deletions											249:	299:	309:			

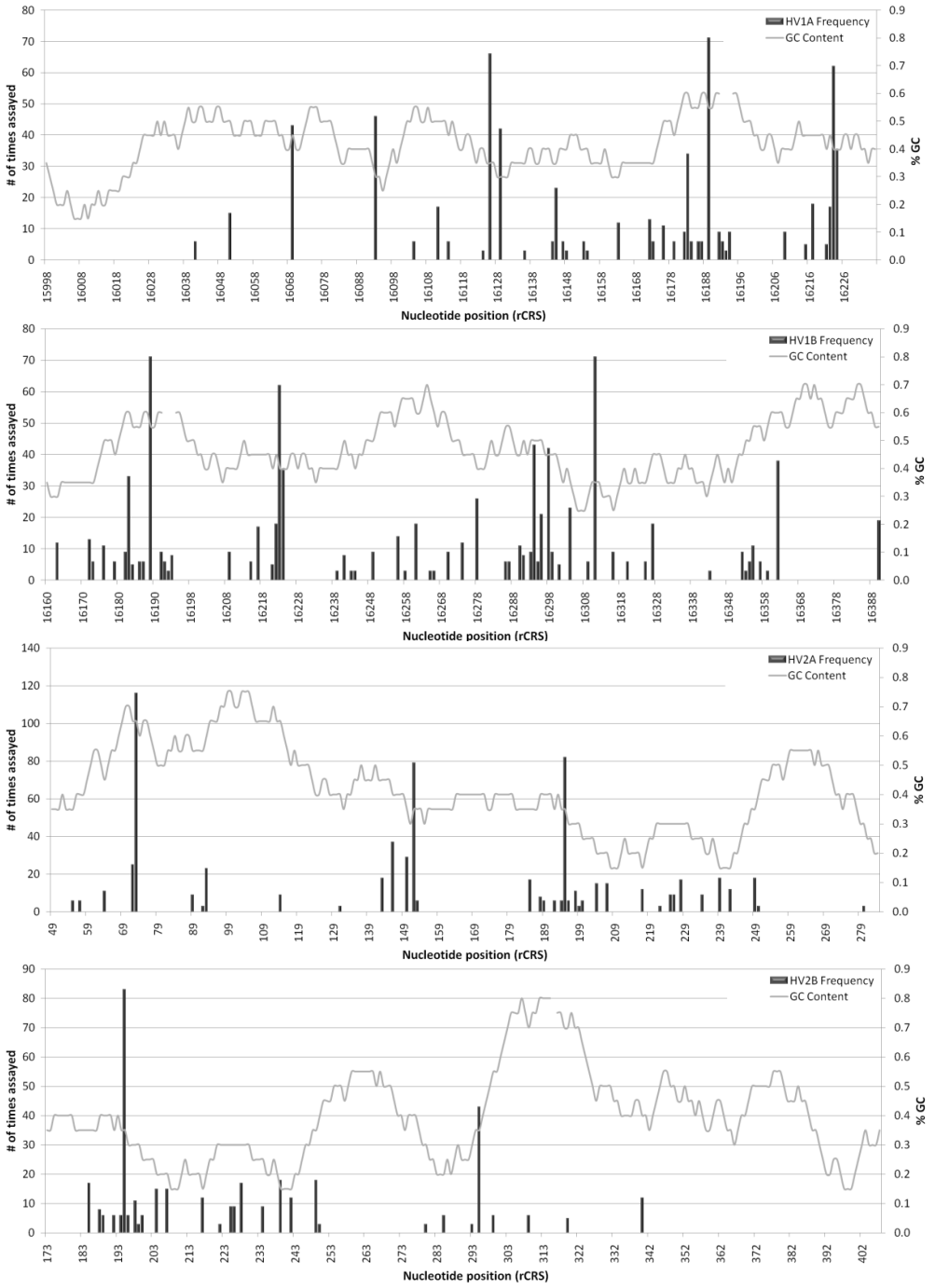


Figure 2.3: A histogram showing the distribution of nucleotide positions within HV1A, HV1B, HV2A and HV2B amplicons (x-axis) and the number of times that sequence polymorphisms at each position were assayed by DHPLC (y-axis). The polymorphisms assayed are broadly distributed throughout both GC and AT rich regions as illustrated by the light gray line which indicates %GC content across each fragment as determined by the surrounding 10 nucleotides.

In HV2B, DHPLC was not able to detect as non-identical, combinations of amplicons that differed only at position 295. This position lies in a narrow stretch of sequence immediately adjacent to a large GC-rich region. Given the thermodynamic stability of this region, a single base mismatch may not sufficiently destabilize the surrounding helix such that an early eluting peak can be discerned. This is a postulate supported by the observation that mixtures of amplicons that possess an additional mismatch in this same region are readily detected.

An inverse relationship was generally observed between the number of positional differences associated with a given pair of non-identical amplicons and the relative heights of the hetero- versus homoduplex peak(s). The height and retention time of a heteroduplex peak is a function of the stability and base sequence of the helix. The more stable a heteroduplex, the more readily it should form relative to the competing homoduplices and thus the greater its peak height (indicative of quantity) on the DHPLC chromatograms. The corollary of this is that the formation of less stable helices is less favored and should be associated with smaller and earlier-eluting peaks on the DHPLC chromatograms. In theory, this could compromise the ability of DHPLC to detect as non-identical some combinations of amplicons that differ at a large numbers of positions. In the current study, however, no examples of such “heteroduplex dropout” were observed.

§ 2 - 3.6 Heteroplasmy Detection

As a naturally occurring combination of two haplotypes, heteroplasmy represents an important but potentially confounding factor for DHPLC-based analyses. Chromatograms obtained from assays of known heteroplasmic samples typically show the presence of early-eluting heteroduplices. In these cases, DHPLC can often provide an indication of the nature of the heteroplasmy. Using either non-denaturing (column temperature = 50°C) or partially denaturing conditions, length heteroplasmy results in a chromatogram characterized by a markedly broadened peak often characterized by multiple shoulders (Figure 2.4). This represents the combination of the various length amplification products and the characteristic shape of such peaks makes it possible to readily screen for heteroplasmy associated with the cytosine-stretches of HV1 and HV2B. Using partially denaturing conditions, point (*i.e.*, base substitution) heteroplasmy can also be readily detected. It is typically indicated by the presence of a defined heteroduplex peak or a significant shoulder in association with the main homoduplex peak.

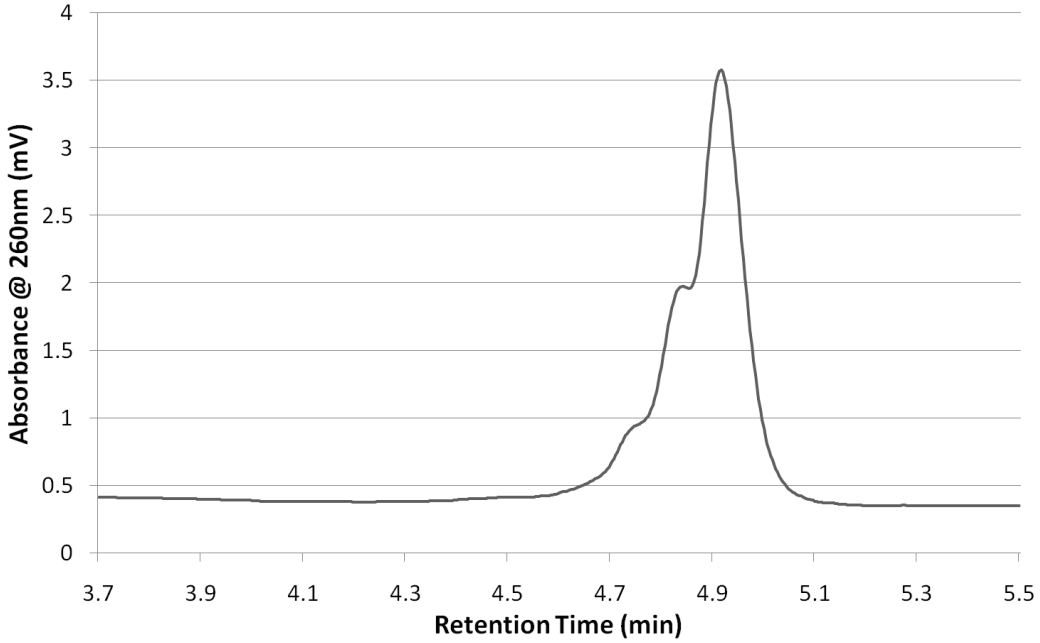


Figure 2.4: An example of length heteroplasmy as detected by DHPLC at a non-denaturing DNASep® column temperature of 50°C. Secondary and tertiary length variants are detected as shoulders prior to the primary length component represented by the major peak.

When analyzing questioned samples, however, it is not possible to reliably distinguish between heteroplasmy and a situational mixture where mtDNA has been contributed by two or more individuals. Even so, the presence of heteroplasmy does not necessarily preclude the use of a heteroplasmic reference sample for comparative sequence analysis by DHPLC. Sequence non-identity in such cases would be indicated by the presence of additional early eluting peaks and/or shoulders that were not evident in the chromatogram generated by cross-hybridization of the heteroplasmic sample with itself. Conversely, the absence of any additional peaks would be consistent with sequence identity.

Heteroplasmy, however, can reduce the discriminatory power of DHPLC in some cases. In the current study, this was most often observed for samples with significant length heteroplasmy. The broad chromatographic peak associated with these samples can obscure the presence of additional heteroduplices formed as a result of cross hybridization between the amplicons present in the known sample and additional amplicons in the questioned sample.

While DHPLC circumvents many of the limitations of alternate approaches to mtDNA screening, it is important to consider very carefully the types of samples for which such an approach might be indicated. Within an mtDNA sequencing laboratory, screening by DHPLC makes it possible to detect samples

that contain mixtures of non-concordant amplicons immediately after PCR amplification and without having to sequence them. For both heteroplasmic and situational mixtures, this approach would enable the analyst to identify potentially challenging samples and mark them for "special handling" – whether that be use of alternate sequencing primers to avoid C-stretch polymorphisms or the application of emerging technologies for resolving mixed samples (Danielson *et al.,* 2005; Andreasson *et al.,* 2006; Danielson *et al.,* 2007).

§ 2 - 3.7 DHPLC as a Presumptive Screen for Sequence Identity

It has been reasonably argued by experienced practitioners in the field that that it is best to avoid the use of mtDNA screening method on limited or irreplaceable evidentiary material (Divne *et al.,* 2005; Melton *et al.,* 2006). A presumptive DHPLC screen for mtDNA sequence identity, however, could serve as a useful tool for investigators in special situations such as the investigation of property crimes. The limited budgets of many law enforcement agencies make it extremely difficult for investigators to justify the expense of mtDNA testing in the majority of criminal offences that do not involve crimes against persons – particularly when there is no assurance *a priori* that the test results will necessarily advance an investigation. A presumptive screen for sequence identity between a suspect and an item of evidence, however, could provide sufficient justification to submit the sample for confirmatory analysis by direct sequencing. Such screening might also help to readily eliminate from consideration such non-

probative samples as hairs consistent with a victim that were recovered from the victim's home or vehicle in the case of a burglary or auto theft. In short, this could help investigators to focus their efforts on the most probative samples and thereby maximize the efficient use of investigative resources.

Employing a presumptive test of mtDNA sequence identity in the manner described above shifts the process of DNA extraction from the dedicated mtDNA sequencing laboratory to the local law enforcement laboratory. This necessitates that additional consideration is given to the handling of these samples. The presence of evidentiary material with large quantities of mtDNA (*e.g.*, blood, saliva and seminal fluids, etc.) in local laboratories can pose a significant risk of cross contamination. Accordingly, an mtDNA sequencing laboratory accepting a DNA extract for analysis would almost certainly require the submission of a co-extracted reagent blank control that could be tested to detect the presence of spurious mtDNA contamination. Similarly, the submission of amplified PCR products for direct sequencing would also need to be accompanied by the appropriate positive and negative PCR controls.

§ 2 - 4 Conclusion

The results of the current study have demonstrated that DHPLC analysis of pair-wise combinations of identical mtDNA amplicons, accurately and reliably produces a single chromatographic peak consistent with sequence identity.

These results were 100% concordant with DNA sequence data. Conversely, in pair-wise combinations of non-identical amplicons, DHPLC successfully detects a diversity of sequence differences throughout the HV1 and HV2 regions. These differences, which include a wide variety of base substitutions as well as insertions/deletions, are typically indicated by the presence of more than a single peak in the resulting chromatogram. As such, DHPLC may have significant forensic utility in several areas. These include: a presumptive test of mtDNA identity between known and questioned samples; a screen for mixed samples prior to direct sequencing and; a preparative tool for the physical fractionation of the individual contributors to an mtDNA mixture prior to sequencing.

Although DHPLC is not a replacement for direct sequencing of mtDNA, it does offer some advantages as a potential screening tool. First, the assay is relatively simple and fast. It uses raw PCR products thereby avoiding the time and expense associated with amplicon cleanup. Following cross-hybridization, each assay takes only seven minutes to run and interpretation of the results is straightforward. DHPLC provides a comprehensive assessment of sequence identity across an entire amplicon without the often challenging task of trying to obtain quality base sequence information immediately adjacent to primer binding sites. Finally, the presence of heteroplasmy, while a potentially confounding variable, does not necessarily preclude comparative sequence analysis by DHPLC.

Compared to alternate mtDNA screening strategies based on oligonucleotide probes or linear arrays, DHPLC consumes less DNA and is not limited by the need to design probes for the detection of known mutations at predetermined polymorphic sites. This reduces the potential for false inclusions and eliminates the need to design custom probes for unique or rare sequence variants. Similarly, DHPLC assays are not subject to the “null” or “blank” results that arise when hybridization of the target sequence is impeded by other nearby polymorphisms. On the contrary, additional sequence variants typically make it easier to detect sequence non-identity between two amplicons.

Forensic laboratory implementation of a commercial DHPLC analysis system can be achieved with minimal training and initial equipment cost of just over $50K. The $0.50/run operating cost for DHPLC analyses is considerably less than that for alternative approaches. Depending on the nature of casework being analyzed and level of throughput, the acquisition of a DHPLC system may be a fiscally viable option for some forensic laboratories as is already the case in the molecular diagnostics arena.

Finally, the current study has also demonstrated that DHPLC can be used to resolve mixtures of non-identical mtDNA amplicons into a series of homo- and heteroduplex peaks. Since these can be differentially eluted in time, it should be possible to physically recover and concentrate the DNA contained in any given

fraction of eluent. Subsequent sequence analysis of the recovered DNA may then provide a basis for determining the specific haplotype of each of the contributors to the mixture. The feasibility of this approach has already been demonstrated (Danielson *et al.,* 2005; Danielson *et al.,* 2007).

§ 2 - 5 Chapter Summary

Denaturing High-Performance Liquid Chromatography (DHPLC) was evaluated as a means of detecting the presence of sequence differences in pair-wise mixtures of non-identical amplicons representing hypervariable regions (HV1 and HV2) of human mitochondrial DNA (mtDNA). The approach is based on the ability to chromatographically distinguish between PCR products on the basis of sequence alone. Using forensically-validated primers sets, optimal assay parameters (*e.g.*, column temperature and acetonitrile gradient) were empirically determined for each of four D-loop amplicons commonly employed by forensic mtDNA laboratories. Following extraction of DNA from 95 unrelated human volunteers, 920 pair-wise combinations were assayed by DHPLC for sequence identity/non-identity. For the 72 combinations of amplicons that were from different individuals but which had identical DNA sequences, DHPLC assay results consistently indicated the presence of sequence identity between the samples. This result was 100% concordant with direct sequencing data for the amplicons tested. For the 849 combinations of amplicons which differed in

sequence, DHPLC correctly detected the presence of sequence non-identity in all but 13 assays to yield 98.5% concordance with direct sequencing. DHPLC can be used to detect a diversity of sequence differences (transitions, transversions, insertions and deletions) at polymorphic sites distributed throughout the HV1 and HV2 regions. The current study demonstrates that DHPLC may have forensic utility as a presumptive test of mtDNA identity between known and questioned samples; as a screen for heteroplasmy or situationally mixed samples prior to direct sequencing and; as a preparative tool for the physical fractionation of the individual contributors to an mtDNA mixture prior to sequencing.

Chapter 3: Validation of Simultaneous Purification and Quantification of PCR Yield by High-Performance Liquid Chromatography on DNASep® Columns

§ 3 - 1 Introduction

High-performance liquid chromatography (HPLC) using an ion-paired reversed-phase column allows for the high-resolution analysis of nucleic acid fragments. In recent years, this technology has been widely employed for the analysis and preparative purification of oligonucleotides; the size fractionation of double-stranded DNA molecules; and the detection of sequence polymorphisms under partially denaturing conditions - a process known as denaturing HPLC (DHPLC). In addition to its use in basic research and molecular diagnostics, the forensic potential of DHPLC has also been recognized. DHPLC has been investigated as a means of rapid gender determination, STR sizing and the typing of Y chromosome biallelic polymorphisms (Underhill *et al.*, 1997; Shinka *et al.*, 2001; Shi *et al.*, 2007). The greatest forensic interest in DHPLC, however, has focused on its potential utility in mitochondrial DNA analysis where it has been used to detect heteroplasmy (Lo *et al.*, 2005; Meierhofer *et al.*, 2005; Jiang *et al.*, 2006), polymorphisms between individuals (Danielson *et al.*, 2005; Wang *et al.*, 2006) and as a method to resolve mtDNA mixtures into identifiable haplotypes (Danielson *et al.*, 2007). Beyond its direct applicability to mtDNA sequence

analysis, DHPLC may also help to facilitate two other tasks in the processing of mtDNA samples, namely PCR product cleanup and yield determination.

Following PCR amplification, mtDNA amplicons are purified to remove unincorporated nucleotides and oligonucleotide primers. PCR cleanup is critical to obtaining quality sequence data and is often accomplished by use of spin columns (*e.g.,* Amicon Centricon-50®) (Millipore Corporation, Billerica, MA) or enzymatic treatment, (*e.g.*, ExoSAP-IT®) (USB Corporation, Cleveland, OH) (Hanke and Wink, 1994; Werle *et al.*, 1994; Dugan *et al.*, 2002). Spin columns effectively remove small DNA fragments, such as primers, but are less efficient at removing larger extraneous amplification products. Additionally, their use has been reported to be prone to inefficient recovery (30% on average) of the target amplicon (Dugan *et al.*, 2002). Treatment with ExoSAP-IT® uses exonuclease I to digest residual single-stranded DNA (*e.g.*, primers and some extraneous amplification products) and shrimp alkaline phosphatase to remove residual dNTPs. This enzymatic approach allows for significantly greater rates of product recovery (78% on average) (Dugan *et al.*, 2002). Both methods, however, are limited by their inability to distinguish between target PCR amplicons and non-specific double-stranded amplification products of similar size (Gabriel *et al.*, 2001). Additionally, casework-type samples may vary in number and/or extraction volume making it difficult to fully automate the PCR product cleanup process on a robotic platform. As a result, analysts may need to set up and

process spin columns or ExoSAP-IT® reactions individually. This can require a significant amount of hands-on time and corresponding reduction in productivity.

The accurate determination of DNA yield following sample amplification/purification is crucial to generating quality sequence data (Kieleczawa, 2006). Excessive DNA input quantities result in sequence electropherograms characterized by signal saturation, inconsistent peak heights and/or premature signal drop off. Conversely, insufficient quantities of input DNA may be associated with signal degradation and decreased S/N ratios. While these effects can sometimes be ameliorated by subsequently adjusting electrokinetic injection times/voltages or by the concentration/dilution of DNA extracts, such remedies are less desirable than simply using an optimal quantity of DNA from the start.

PCR yield can be quantified by a number of approaches including UV spectroscopy, gel electrophoresis, or microchip capillary electrophoresis all of which have a number of shortcomings. UV spectroscopy cannot distinguish between an amplified target and residual primers or spurious PCR amplification products. As a result, the amount of amplified target DNA can be easily overestimated. Agarose “yield gels”, which are used in many forensic laboratories are moderately labor intensive and require that samples be compared to a quantification ladder using either the eyes of a trained analyst or

pixel counting software. The Agilent 2100 Bioanalyzer Lab Chip capillary electrophoresis system (Agilent Technologies, Palo Alto, CA) offers greater accuracy and precision but requires investment in expensive dedicated equipment, higher consumable costs per sample and a number of sample handling steps.

The ability to fractionate by size, quantify and physically recover macromolecules is fundamental to chromatographic techniques (Kalnoski *et al.*, 1991; Warren and Doniger, 1991; Henninger *et al.*, 1993; Zeillinger *et al.*, 1993; Katz, 1996) including ion-pair reversed-phase chromatography. Purification of DNA fragments by HPLC should make it possible to recover target amplicons in a manner suitable for dye-terminator labeling reactions while circumventing the limitations of existing reaction cleanup approaches. Additionally, given that chromatographic peaks reflect the quantity of DNA eluted from the HPLC column, the height and area of a target DNA peak should provide an accurate means of assessing PCR yield for a target amplicon. The current study, therefore, sought to evaluate the potential for use of a commercial HPLC system (WAVE® 3500HT Nucleic Acid Fragment Analysis System, Transgenomic, Inc., Omaha, NE) to quantify PCR product yield and simultaneously purify target amplicons for downstream applications such as DNA sequencing. In order to demonstrate the efficacy of such an approach, the linearity of DNA quantification by HPLC was assessed across a range of amplicon sizes and concentrations of potential utility

to forensic analysts. Similarly, the suitability of HPLC as a means of amplicon purification was assessed in comparison to existing PCR product cleanup methods employed by forensic laboratories (*i.e.,* ExoSAP-IT® and Amicon Centricon-50® spin columns).

Given the potential utility of HPLC as a means of detecting and fractionating mtDNA mixtures (Danielson *et al.*, 2007), the use of the same platform for PCR yield quantification and reaction cleanup is attractive. This approach could help to save valuable analyst time, increase productivity and streamline the processing of casework-type samples for mtDNA sequencing.

§ 3 - 2 Materials and Methods

§ 3 - 2.1 DNA Quantification Standards

A commercial DNA quantification standard (MassRuler™ Express DNA Ladder, LR Reverse, Lot# 00000201) (Fermentas Life Sciences, Hanover, MD) was used to assess the correlation between DNA quantity and chromatographic peak height/area. This commercial standard is produced in an ISO9001 certified facility and the absence of nucleases is confirmed by a direct nuclease activity assay. The DNA ladder consisted of a mixture of six chromatography-purified and spectrophotometrically quantified DNA fragments (100, 200, 300, 500, 700 and 1000bp) at a stock concentration of 10ng/µL, 7ng/µL, 5ng/µL, 3ng/µL, 2ng/µL and 1ng/µL, respectively. The stock concentration and a series of 2-fold serial

dilutions (1:2 to 1:32768) were prepared in nuclease-free water to yield a series of quantification standards ranging from 30fg/µL to 10ng/µL.

§ 3 - 2.2 HPLC Quantification Assays

All HPLC quantification assays were performed in quintuplicate using a DNASep® analytical column packed with alkylated poly(styrenedivinylbenzene) resin (Huber *et al.*, 1993) installed in a WAVE® 3500HT Nucleic Acid Fragment Analysis System (Transgenomic Inc., Omaha, NE). Stock and each of the serially-diluted quantification standards were injected (20µl/injection) at a 0.9ml/min flow rate and allowed to bind the DNASep® column at 50°C in the presence of 0.1M triethylammonium acetate (TEAA) pH 7.0 as an ion pairing reagent. DNA fragments were sequentially eluted on the basis of size using a gradient of increasing acetonitrile produced by differential mixing of buffer A (0.1M TEAA) and buffer B (0.1M TEAA:25% ACN). Starting with a mixture of 63% buffer A and 37% buffer B, the optimal gradient for the quantification assays employed stepwise increases to 42.1%, 58.8%, 63.1%, 65.2%, 66.3% and 67.1% of buffer B in 3.5 min., 2.0 min., 1.6 min., 1.5 min., 1.5 min., and 1.4 minutes, respectively. Eluted DNA fragments were detected by UV absorbance (260nm) and fluorescence (ex. 490nm; em. 520nm). Fluorescence detection employed a proprietary DNA intercalating dye (WAVE® Optimized HS staining solution I) injected downstream of the UV detector using an inline high sensitivity accessory (HSX) pump (Transgenomic, Inc.). Due to signal saturation, quantification

standards at ≥3.75ng/µL DNA were not assayed by fluorescence. The peak height (mV) and peak area (mV*msec) were determined using the Transgenomic Navigator™ peak analysis software and DNA quantities based on peak height and area were determined by regression analysis.

§ 3 - 2.3 Mitochondrial DNA Extraction and Purification

This research was conducted in compliance with U.S. Federal Policy for the Protection of Human Subjects (56 FR 28003). Casework-type samples were prepared from buccal swabs, peripheral blood, saliva, semen and a variety of hair (0.5cm of shaft only) samples collected from individuals who had provided informed consent to participate in the study. To test the potential impact of different substrates commonly encountered in a forensic context, DNA extracts were prepared from biological stains (10µL peripheral blood, semen or saliva) applied to nylon fabric, synthetic carpeting, leather, unpainted wood or wallboard. To test the potential impact of environmental insult, DNA extracts were prepared from 10µL aliquots of peripheral blood, semen or saliva deposited on Pur-Wraps® sterile cotton tipped applicators (Hardwood Products, Guilford, ME) that had previously been dipped in a slurry of top soil / nanopure water (50:50 w/v), laundry detergent, used motor oil, gasoline, 1M sodium hydroxide or 1M acetic acid and allowed to dry for 2 hours at room temperature. Finally, DNA was extracted from head hairs (natural, dyed dark brown or permed), axial hair, pubic hair, a vaginal swab and aged human bone material (bones

generously provided by Dr. Randall Skelton, University of Montana, Missoula, MT). Bone samples were cleaned twice in 5% Tergazyme™, sonicated for 30-45 min., rinsed in 95% EtOH, dried overnight at room temperature and then pulverized with a SPEX CertiPrep 6850 Freezer/Mill in accordance with manufacturers' recommended protocol for small bone samples. Three, 4-min. cycles (2min grinding period, 2min cooling period) yielded a fine bone powder, 200mg of which mixed with 700µL EDTA (0.5M, pH 8.5) and incubated at 37°C for 24 - 48 hours. Total human DNA was then extracted from all samples using the EZ1 DNA tissue kit on the BioRobot EZ1 DNA extraction robot (Qiagen Inc., Valencia, CA); eluted into a final volume of 200µL of nuclease-free water. All extracts were stored at -20°C until used.

Four forensically relevant regions of the human mitochondrial control region (*i.e.,* HV1A, HV1B, HV2A and HV2B) were amplified using validated PCR primers (Wilson *et al.*, 1995; LaBerge *et al.*, 2003); 2.25U Ampli*Taq* GOLD® DNA polymerase (Applied Biosystems) supplemented with 0.25U *Pfu* DNA polymerase (Stratagene, La Jolla, CA); Ampli*Taq* GOLD® Buffer with $MgCl_2$ at a final concentration of 2mM (Applied Biosystems); 10nmol of each dNTP (Stratagene) and 10µL of an approximately 10pg/µL human genomic DNA extract. Amplifications were performed on a GeneAmp® 9700 thermocycler (Applied Biosystems) with an initial denaturation at 95°C for 10 minutes, followed by 32

cycles of 95°C for 20 seconds, 60°C for 30 seconds and 72°C for 45 seconds. The final extension at 72°C was for 15 minutes.

Amplified PCR products were purified in preparation for DNA sequencing using one of three methods: Amicon Centricon-50® centrifugal filtration (Millipore Corporation, Billerica, MA), ExoSAP-IT® (USB Corporation, Cleveland, OH), or HPLC purification on the WAVE® 3500HT Nucleic Acid Fragment Analysis System. Column purification employed two washes (2ml each of nuclease-free H_2O) using the Amicon Centricon-50® spin columns per the manufacturer's protocol. ExoSAP-IT® treatment was performed per manufacturer's protocol using 2µL of proprietary ExoSAP-IT® reagent / 5µL amplified DNA product and incubation at 37°C and 80°C for 15 minutes each. HPLC purification employed 5µL and 20µL injection volumes and an optimized gradient (56.5% to 65.5% buffer B increase in 4.5 minutes) for HV1A, HV2A and HV2B and (56.3% to 65.3% buffer B increase in 4.5 minutes) for HV1B. Samples were eluted at 0.9ml/min flow rate, detected by UV at 260nm and quantified based on a MassRuler™ Express DNA Ladder standard curve. Samples containing >200ng were diluted and reassayed until the chromatographic peak was within the bounds of the standard curve. Eluted peaks were captured using an automated fraction collector (Transgenomic, Inc.); dried by vacuum centrifugation for 30 minutes at 50°C followed by a 20-minute cool down to ambient on a CentriVap® Vacuum

Concentrator (Labconco Corporation, Kansas City, MO). Samples were resuspended in nuclease-free H_2O; and stored frozen until DNA sequencing.

§ 3 - 2.4 Mitochondrial DNA Sequencing

Dye-terminator sequencing was performed using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Labeling reactions (10µl total volume) were prepared in accordance with the manufacturer's protocol using 0.8ng of template DNA. Dye-terminated products were purified by Performa® DTR V3 96-well short plate purification columns (Edge Biosystems, Gaithersburg, MD) and resolved on the PRISM® 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocols using the POP-6™ polymer and 47cm x 50µm capillaries (Applied Biosystems). The raw electrophoretic traces were analyzed using the KB Basecaller (Applied Biosystems) together with the appropriate dye set mobility file for the v1.1 kit. Appropriate positive, negative controls and reagent blank controls were carried through the entire sample handling process to monitor for contamination and reagent integrity.

The resulting sequence data were displayed using both the Sequencing Analysis Software v.5.1.1 (Applied Biosystems) and Sequencher™ v4.2 DNA analysis software (Gene Codes Corp, Ann Arbor, MI). QV scores were automatically calculated by the Sequencing Analysis Software using the equation $QV=-10\log_{10}(Pe)$ where *Pe* is the probability of an error in a base call. While QV

scores range from 0 to a theoretical maximum of infinity, the sequence analysis software only reports QV scores within a 1-99 range. In general, QV scores of 1-20 at pure base positions generally indicate a low-quality base call while those of 20-50 or higher (*i.e.*, QV20+) indicate a high-quality base call. Mixed base positions typically have lower QV scores than non-mixed positions Sequence quality was evaluated on the basis of both QV20+ Scores and Trace Scores – the latter being the average of all QV scores across the entire post-trimmed sequence. (Biosystems, 2003).

§ 3 - 3 Results

§ 3 - 3.1 Quantitation of PCR Yield on a DNASep® Column

Using a non-denaturing column temperature of 50°C, double-stranded DNA can be readily fractionated to yield a trace in which the height and area of a chromatographic peak reflect the quantity of eluted DNA. To evaluate the accuracy and reproducibility of using these peak parameters to calculate PCR yield, a commercial double-stranded DNA quantification standards and a series of serial dilutions were fractionated by HPLC using a DNASep® column. Each of the resulting chromatographic traces was characterized by the presence of six DNA peaks representing double-stranded fragments ranging in size from 100bp - 1000bp, as described above. Based on the quantification standards used, the lower limit (Mean±SD) for reproducible UV and fluorescence detection was

1.85±0.56ng and 30.9±1.4pg, respectively based on automated peak detection by the Navigator™ analysis software (Transgenomic, Inc.).

Figure 3.1 displays the regression between an injected DNA quantity and either the height or area of the resulting chromatographic peak based on UV detection at 260nm. The linear regressions for each size standard based on peak height (figure 3.1A) had coefficients of determination which ranged from 0.960 (1000bp fragment) to 0.999 (100bp and 200bp fragments). The linear regressions for each size standard based on peak area (figure 3.1B) had coefficients of determination ranging from 0.908 (1000bp fragment) to 0.999 (100bp, 200bp and 300bp fragments).

As presented in figure 3.2, a second degree polynomial regression was found to best delineate the relationship between an injected DNA quantity and the height or area of the resulting chromatographic peak based on fluorescence detection data. The coefficients of determination for the polynomial regressions based on peak height for each size standard (figure 3.2A) ranged from 0.991 (200bp fragment) to 0.999 (700bp and 1000bp fragments). When based on peak area (figure 3.2B), the coefficients of determination for the resulting polynomial regressions were 0.998 (100bp and 200bp fragment) to 0.999 (300bp, 500bp, 700bp and 1000bp fragments).

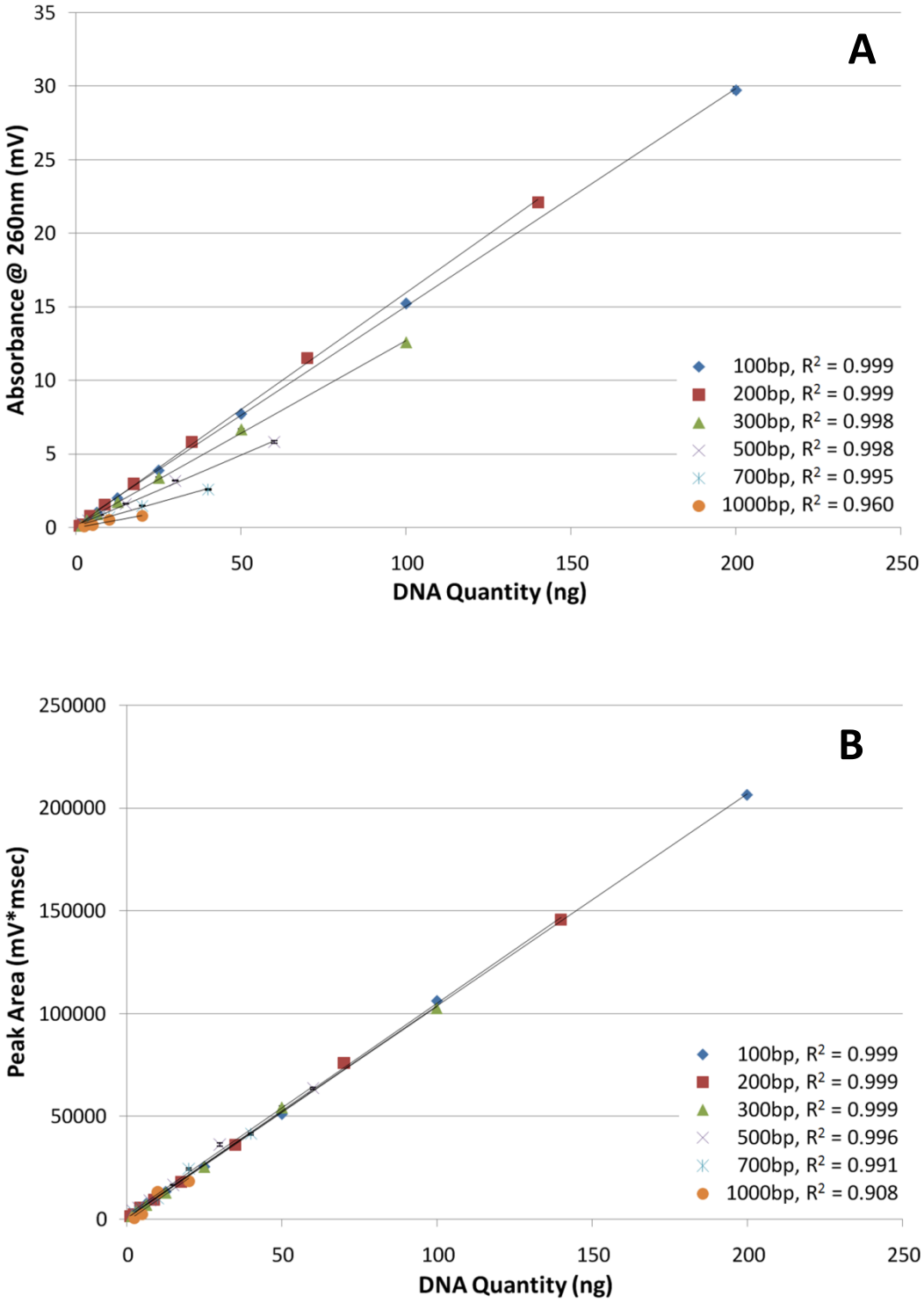


Figure 3.1: Linear regression analysis of (A) peak height and (B) peak area data collected by UV detection at 260nm.

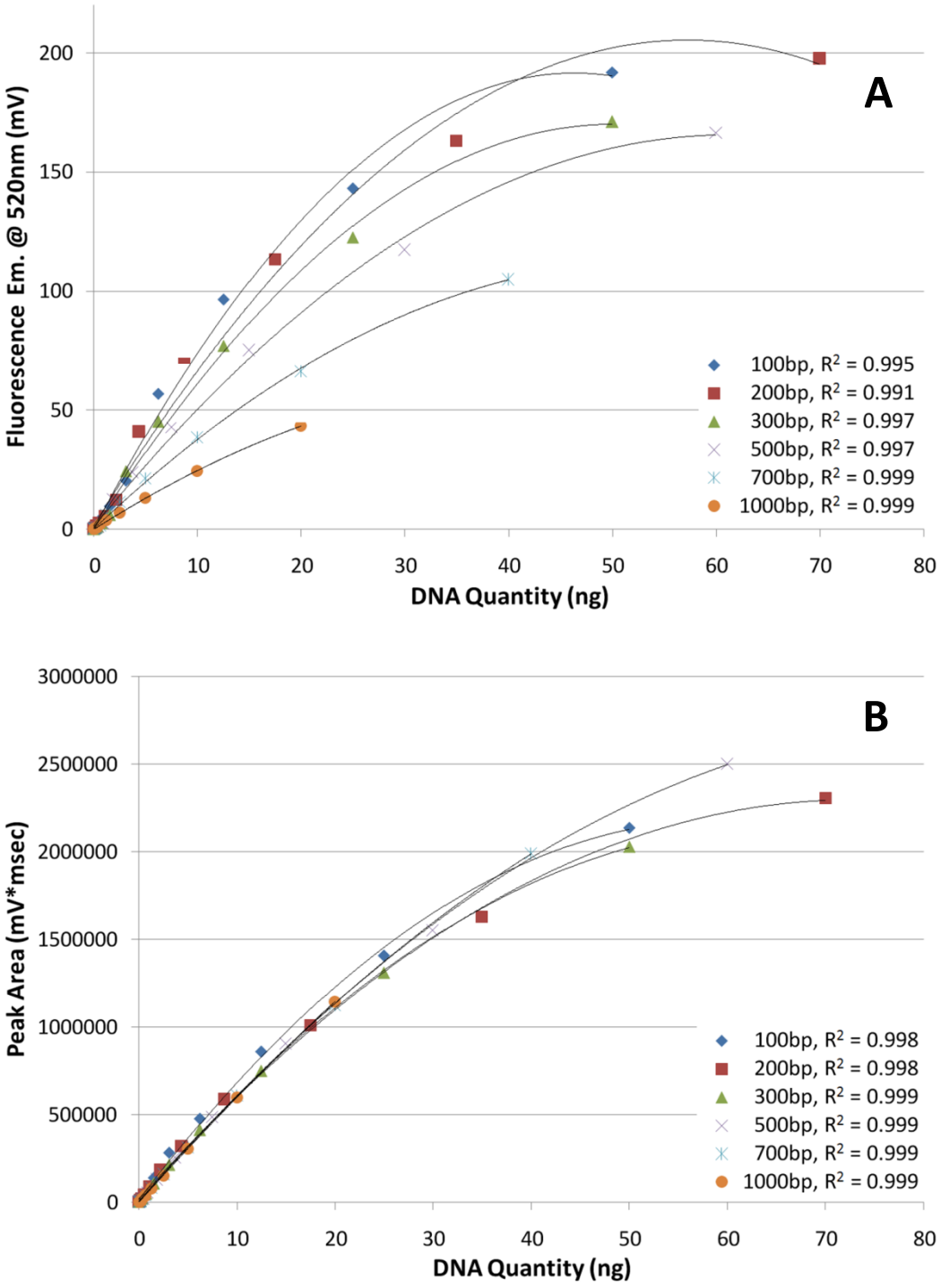


Figure 3.2: A second degree polynomial regression analysis of (A) peak height and (B) peak area data collected by fluorescence detection using a proprietary DNA intercalating dye and the HSX high sensitivity accessory.

§ 3 - 3.2 Efficiency of PCR Product Recovery and Sequence Quality

To determine the efficiency of PCR product recovery associated with DNASep® column purification, 100ng, 70ng, 50ng, 30ng, 20ng and 10ng of the commercial quantification standards (100bp, 200bp, 300bp, 500bp, 700bp and 1000bp, respectively) were assayed in triplicate. For each size fragment, the DNA represented by the resulting chromatographic peak was detected by UV, physically recovered by an automated fraction collector, dried and then reassayed by HPLC on a DNASep® column and quantified by comparison to the MassRuler™ Express DNA Ladder standard curve. The average±SD recovery efficiencies were found to be 72.68±2.6%, 70.05±7.9%, 63.58±5.9%, 69.6±7.7%, 64.9±7.2%, 60.76±8.3% for the 100bp, 200bp, 300bp, 500bp, 700bp and 1000bp fragments, respectively.

To compare the quality of sequence data obtained from templates purified by HPLC on a DNASep® column versus alternate cleanup methods currently used in many forensic laboratories, mtDNA was amplified from extracts of five dried 10µL spots of whole blood. Aliquots (40µL) of the crude post-amplification product were purified by either HPLC using a DNASep® column, ExoSAP-IT® treatment or Amicon Centricon-50® spin columns. Detail from examples of sequencing electropherograms generated from dye-terminator labeling of 0.8ng of purified PCR product are presented in figure 3.3. All three post amplification cleanup methods yielded full-length sequences (257 bp) with

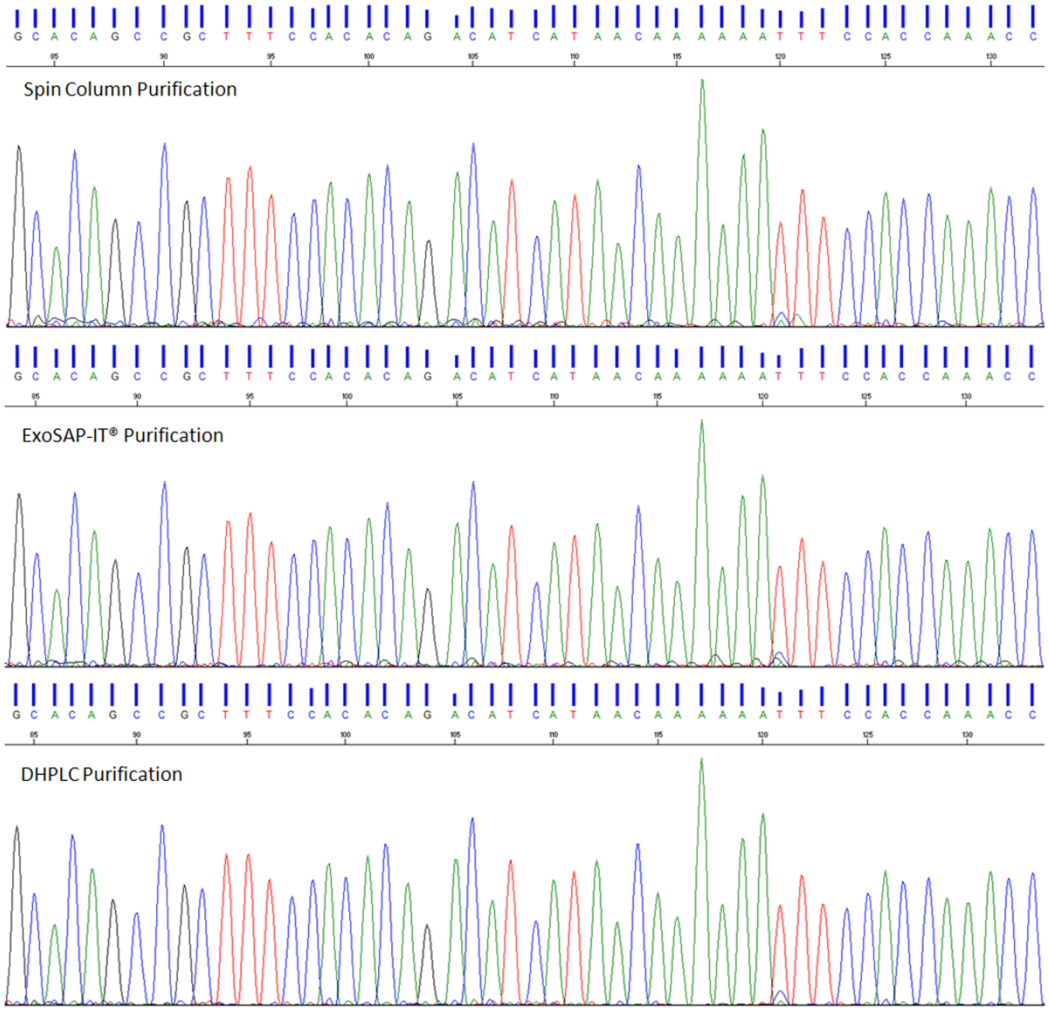


Figure 3.3: Examples of sequencing electropherograms generated using the BigDye® Terminator v1.1 Cycle Sequencing Kit and 0.8ng input DNA. HPLC purification shows higher signal-to-noise ratio than either Spin column or ExoSAP-IT® purification methods. Y-axis (max 2000 RFU) is the same for all three purification methods.

high quality base calls. Averaged across the five replicate sequencing reactions for each purification method, the overall trace scores (i.e., average QV for all bases) were 55.6, 54.6 and 54.4 and the QV20+ scores were 252.4, 251.2, and 251.2 for the samples purified by HPLC, ExoSAP-IT® treatment, and Amicon Centricon-50® spin columns, respectively. The ratio of sequence signal to baseline noise across each trace was also assessed as an indicator of sequence quality. Averaged across the five replicate sequencing reactions for each purification method, total baseline noise accounted for 4.86±0.69%, 6.04±1.68% and 6.52±0.54% of total signal for the samples purified by HPLC, ExoSAP-IT® treatment, and Amicon Centricon-50® spin columns, respectively.

§ 3 - 3.3 Casework type Samples

A diversity of casework type samples (Table 3.1) were employed to assess the accuracy of using HPLC on a DNASep® column to simultaneously quantify and purify post-amplification products in preparation for DNA sequencing. For each sample, the amount of post-amplification product (ng) was determined based on area of the chromatographic peak. This value was used to determine the volume of sterile water in which to resuspend the purified mtDNA fragment (1.0µL ddH_2O per 0.8ng of DNA) for dye-terminator labeling. The robustness of this approach was evaluated on the basis of the quality of the resulting sequence data. Pristine reference type samples were prepared from dried peripheral blood or buccal cells on sterile cotton swabs. Recognizing that PCR amplification of

Table 3.1: Summary of casework-type samples used in the study. Samples were isolated from a variety of substrates and exposed to a variety of environmental degradation. DNA yield depended on HPLC injection volumes as well as inhibition to amplification. QV and QV20+ scores obtained were highly consistent.

Item	Source	Substrate	Contaminant	Fragment	Injection (µL)	Quantity (ng)	QV	QV20+	LOR	Mixture
1	Buccal	Cotton Swab	None	HV2B	5	8.6	59	256	257	N
2	Buccal	Cotton Swab	None	HV1B	5	12.6	49	241	251	N
3	Blood	Cotton Swab	None	HV1A	5	67.7	49	244	258	N
4	Blood	Cotton Swab	None	HV2A	5	81	49	250	258	N
5	Blood	Denim	None	HV2A	20	162.6	46	249	258	N
6	Blood	Leather	None	HV1B	20	256.6	45	238	251	N
7	Blood	Leather	None	HV2A	20	63.1	46	249	258	N
8	Blood	Leather	None	HV1B	20	431.9	50	243	251	N
9	Blood	Wallboard	None	HV1B	20	410	49	245	251	N
10	Blood	Wood	None	HV1B	20	354.3	44	233	251	N
11	Blood	Wood	None	HV2A	20	159.8	47	249	258	N
12	Blood	Wood	None	HV1B	20	370.4	50	244	251	N
13	Saliva	Carpet	None	HV1B	20	443	47	241	251	N
14	Saliva	Leather	None	HV1B	20	419	48	240	251	N
15	Saliva	Wallboard	None	HV1B	20	348.8	47	242	251	N
16	Saliva	Wood	None	HV1B	20	415.2	48	240	251	N
17	Semen	Carpet	None	HV1B	20	274.9	44	235	251	N
18	Semen	Nylon	None	HV1B	20	203.4	46	240	251	N
19	Blood	Cotton Swab	Detergent	HV1B	20	420.2	45	242	251	N
20	Blood	Cotton Swab	Detergent	HV2A	20	140.5	46	247	258	N
21	Blood	Cotton Swab	Detergent	HV1B	20	334.5	48	240	251	N
22	Blood	Cotton Swab	Gasoline	HV1B	20	413	45	237	251	N
23	Blood	Cotton Swab	Gasoline	HV2A	20	189.8	46	250	258	N
24	Blood	Cotton Swab	HAC	HV1B	20	462.6	46	245	251	N
25	Blood	Cotton Swab	HAC	HV1B	20	364.7	49	240	251	N
26	Blood	Cotton Swab	NaOH	HV2A	20	33.9	44	242	258	N
27	Blood	Cotton Swab	Soil	HV2A	20	11.5	49	244	251	N
28	Blood	Cotton Swab	Used motor oil	HV1B	20	365.8	45	243	251	N
29	Blood	Cotton Swab	Used motor oil	HV1B	20	427.6	50	242	251	N
30	Saliva	Cotton Swab	Gasoline	HV1B	20	458.5	47	241	251	N
31	Semen	Cotton Swab	NaOH	HV1B	20	120.5	47	240	251	N
32	Semen	Cotton Swab	Soil	HV1B	20	31.4	44	238	251	N

reference samples is typically robust, only 5µL injection volumes were used for HPLC purification. The amount of purified post-amplification product ranged over nearly an order of magnitude from 8.6ng (Item 1, buccal swab) to 81.0ng (Item 4, blood swab). The resulting sequence data were characterized by QV20+ scores of 241 to 256 for sequences having 251 to 258 called bases and high trace scores of 49 to 59 representing an average basecalling confidence level of 99.9987% to 99.99987% across the post-trimmed sequence.

The potential impact of a diversity of substrates (i.e., carpet, blue denim, leather, nylon fabric, wallboard and wood) from which biological stains (blood, saliva, and semen) could be collected was also tested. Not knowing the extent to which a given substrate might influence PCR amplification, 20µL injection volumes were used for HPLC purification. Following quantification and purification by HPLC, the recovered post-amplification products were labeled and sequenced as described above. All samples amplified successfully with yields ranging from 63.1ng (Item 7, blood on leather) to 443ng (Item 13, saliva on carpeting). Regardless of the yield of the PCR reaction, the resulting sequence data generated from DNA quantified and purified by HPLC were of uniformly high quality. Trace scores ranged from 44 to 50 (i.e., 99.996% to 99.999% confidence) and QV20+ scores ranged from 233 to 249 for sequences having 251 to 258 called bases.

To assess the potential impact of common environmental insults (i.e., laundry detergent, gasoline, used motor oil, soil, 1M sodium hydroxide and 1M acetic acid), biological stains intermixed with these contaminants were extracted and amplified. As before, 20µL injection volumes were used for HPLC quantification and purification. All samples amplified and yielded from 11.5ng (Item 27, blood with soil) to 462.6ng (Item 24, blood with acetic acid). Sequencing of the recovered post-amplification products yielded high quality results. Trace scores ranged between 45 and 50 (i.e., 99.997% to 99.999% confidence) and QV20+ scores were between 237 and 250 for sequences having a total of 251 to 258 called bases.

To test the utility of HPLC quantification and purification with challenging samples more typically encountered in a forensic mtDNA context, a diversity of hair shafts (natural, dyed and permed), aged bone samples and vaginal swabs were also tested (Table 3.2). Vaginal swabs generally yielded the highest quantities of post amplification product on average and the resulting sequence quality was excellent with trace scores between 46 and 49 (i.e., 99.9974% to 99.9987% confidence) and QV20+ scores of 248 to 251 out of 257 to 258 bases called. As expected, amplification yields with hair and bone samples were frequently lower than with DNA extracts of blood, saliva or semen. Using a 20µL injection volume, the amplification yield from hair shaft extracts range from 32.9ng (Item 49, permed head hair) to 394.3ng (Item 46, untreated head hair).

Table 3.2: Summary of hair, bone and vaginal swab samples used to validate HPLC as a tool for single step purification and quantification. HPLC and dye-terminator sequencing detected mixtures in 4 of 5 bone samples and length-heteroplasmy in 6 of 19 hair samples.

Item	Source	Substrate	Contaminant	Fragment	Injection (µL)	Quantity (ng)	QV	QV20+	LOR	Mixture
33	Bone	None	Aged	HV2A	20	2.7	48	243	256	Y
34	Bone	None	Aged	HV2A	20	53.4	47	245	256	Y
35	Bone	None	Aged	HV2A	20	26.7	47	246	258	Y
36	Bone	None	Aged	HV2A	20	40.1	46	248	258	Y
37	Bone	None	Aged	HV2A	20	108.7	47	251	258	N
38	Axial hair	None	None	HV1A	20	178.7	48*	228	258	LH
39	Head hair	None	Dyed	HV1A	5	53.4	50	242	258	N
40	Head hair	None	Dyed	HV1A	5	20.1	47	245	258	N
41	Head hair	None	Dyed	HV2B	5	59.9	48	252	257	N
42	Head hair	None	Dyed	HV2B	5	14.2	41*	194	257	LH
43	Head hair	None	Dyed	HV1A	5	46.8	44	241	258	N
44	Head hair	None	None	HV2B	5	54.4	48	252	257	N
45	Head hair	None	None	HV1A	20	50.9	50	248	258	N
46	Head hair	None	None	HV1A	20	394.3	48	245	258	N
47	Head hair	None	None	HV1A	20	214.6	46	249	258	N
48	Head hair	None	None	HV2B	20	68.5	47	246	257	N
49	Head hair	None	Permed	HV1A	20	32.9	46*	238	258	LH
50	Pubic hair	None	None	HV1A	20	71.6	48*	238	258	LH
51	Pubic hair	None	None	HV1A	5	19.5	57	245	258	N
52	Pubic hair	None	None	HV2B	5	18.5	43*	209	257	LH
53	Pubic hair	None	None	HV1A	5	49.7	49	247	258	N
54	Pubic hair	None	None	HV1A	5	49.1	38	239	258	LH
55	Pubic hair	None	None	HV2B	5	46.8	48	252	257	N
56	Pubic hair	None	None	HV2B	5	43.8	47	252	257	N
57	Vaginal swab	Cotton Swab	None	HV1A	20	492.3	49	250	258	N
58	Vaginal swab	Cotton Swab	None	HV1A	20	202.8	47	249	258	N
59	Vaginal swab	Cotton Swab	None	HV2B	20	225.4	49	251	257	N
60	Vaginal swab	Cotton Swab	None	HV2B	20	146.5	46	248	257	N

*QV scores were determined until the point of length heteroplasmy (LH)

Using a 5µL injection volume, the yield of HPLC purified DNA ranged from 14.2ng (Item 42, dyed head hair) to 59.9ng (Item 41, dyed head hair). The lowest yields were seen with bone samples where a 20µL injection volume yielded as little as 2.7ng of amplified DNA (Item 33, aged bone) for labeling. Regardless of the tissue employed, the amplification yield or the injection volume purified, all post-amplification products that were quantified and purified by HPLC yielded high quality sequence data characterized by trace scores which, except for heteroplasmic samples, ranged from 44 to 57 (i.e., 99.996% to 99.9998% confidence). Similarly, the QV20+ scores for the same samples ranged from 241 to 252 for sequences in which 256 to 258 base calls were made. Although sequence results from four of five aged bone samples presented as mixtures, the overall quality of these sequences was still high with trace scores of 46 to 48 (i.e., 99.9974% to 99.9984% confidence) and QV20+ scores 243 to 248 out of 256 to 258 base calls.

§ 3 - 3.4 HPLC Purification and Length Heteroplasmy

Mitochondrial DNA length heteroplasmy most commonly occurs in association with the C-stretch motifs in HV1 and HV2B. Length heteroplasmy arising from insertion/deletion events at other positions may also be encountered when processing either reference or casework type samples. In the current study, length heteroplasmy was readily identified during HPLC

purification and quantification. As illustrated in figure 3.4, each single-base length variant forms a clearly discernible shoulder adjoining the chromatographic peak of the primary amplification product (figure 3.4, buccal swab). In cases where the quantity of a length variant approaches that of the major product, a separate chromatographic peak forms before or after the major product peak with shorter or longer variants, respectively (figure 3.4, blood stain). Non-length (*i.e.*, substitution) variants were not distinguishable from the major product under non-denaturing conditions used for post-amplification quantification and purification.

Using casework-type samples, length heteroplasmy was observed in the chromatographic traces for either the HV1A or HV2B fragment of 6 out of 19 hairs tested. In each case, the resulting sequence electropherograms confirmed the length heteroplasmy. In no case was heteroplasmy detected by sequencing but not by HPLC. In terms of sequence quality, heteroplasmic samples tended toward slightly lower trace scores which ranged from 38 to 48 (*i.e.*, 99.984% to 99.9984% confidence) even for data outside of the region directly impacted by the length polymorphism. The same was seen in the QV20+ scores which ranged from 194 to 239 for sequences where 257-258 base calls were made.

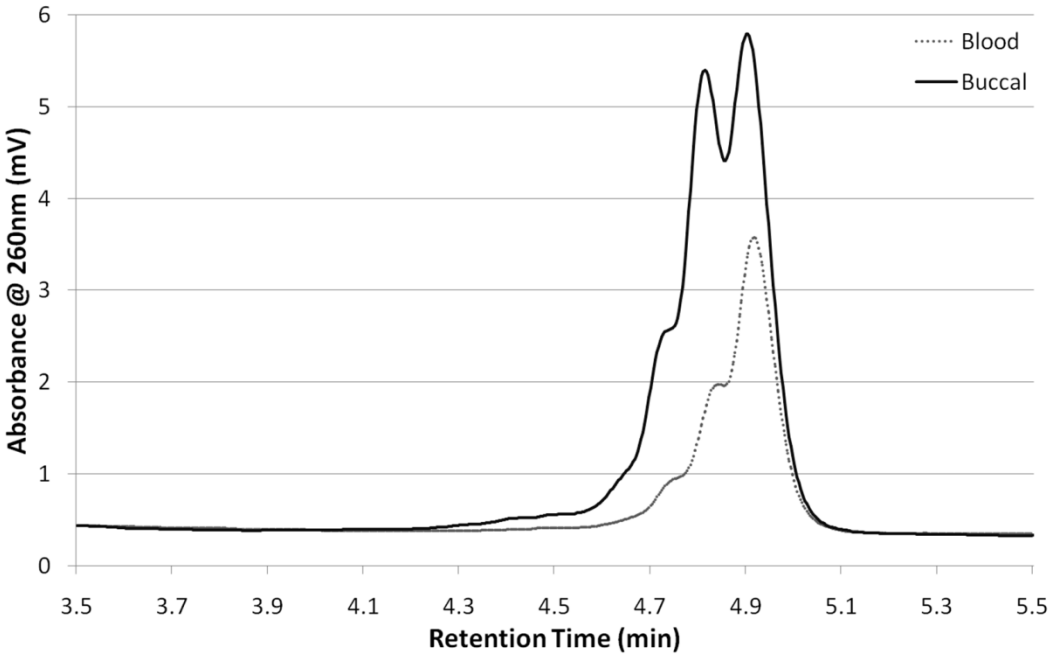


Figure 3.4: An example of length heteroplasmy as detected by HPLC at a non-denaturing DNASep® column temperature of 50°C. Secondary and tertiary length variants are detected as shoulders prior to the primary length component.

§ 3 - 4 Discussion

Reaction cleanup and yield quantification prior to dye-terminator labeling is an essential part of obtaining accurate and high quality sequencing electropherograms. Validation of a highly automated system for quantification, purification and characterization (*i.e.,* length heteroplasmy) of amplified PCR products has, therefore, been conducted in order to streamline the processing of casework-type mtDNA samples. Results of validation studies presented, show that quantification and purification of crude PCR products can be performed in a highly automated manner while also differentiating target PCR amplicons from non-specific double-stranded amplification products.

Quantitation results of PCR yield by HPLC under non-denaturing conditions are highly consistent across all six tested fragment ranges for both UV and fluorescence detection. While reported detection limits were determined using the Navigator™ analysis software's automated peak detection and analysis, manual determination of peak appearance and analysis of peak height and peak area yielded detection limits in accordance with manufacturer's specifications (UV: approx. 0.5ng and fluorescence: <10pg, www.transgenomic.com, data not shown). Regression analyses of both peak height and peak area show that determination of DNA quantity is highly consistent, although determination of peak area is more consistent across multiple fragment sizes. With the occurrence of a secondary peak (due to length heteroplasmy of mtDNA samples) or peak

broadening, the use of peak height can underestimate the total quantity of PCR product present in the sample. Realizing that peak height determination is not always possible, due to various length variants within the same amplification product, quantity determination using peak area is, therefore, a more reliable method for quantity determination.

Efficiency of PCR product recovery using DNASep® column purification was determined to be similar to results reported for ExoSAP-IT® and the QIAquick PCR Purification columns in Dugan *et al.,* The benefit, however, is that DNASep® column purification yields information regarding PCR efficiency (*e.g.*, target quantity, presence of spurious amplification products, length variants, etc.) in a single fully automated step.

Sequence quality obtained by dye-terminator labeling using the BigDye® v1.1 Cycle Sequencing Kit for dried blood spots was slightly higher when purified by HPLC on a DNASep® column versus alternate cleanup methods. All purification methods yield high quality electropherograms (with average QV20+ scores above 250 for a 257bp product), although only the HPLC purification allows for definite determination of low level heteroplasmy at position 292 as compared to the Cambridge Reference Sequence (Figure 3.3). This is due to the overall greater signal-to-noise ratio of basecalls for the HPLC purified samples.

The quality of sequencing electropherograms extends into the casework type samples employed in this study. Results obtained from a diversity of casework type samples employed in this study and the overall high quality scores (QV and QV20+) of electropherograms obtained show that purification of crude PCR reactions and simultaneous determination of PCR yield by HPLC is a reliable and consistent platform for obtaining quality mtDNA sequencing electropherograms. Additionally, the ability to accurately determine and standardize the DNA input quantity and not having to subsequently adjust sequencing run parameters to obtain good quality electropherograms saves valuable analyst time and increases productivity.

The ability of HPLC to detect length variants expands on the ability of purification and quantification of crude PCR products. Traditionally, this critical information is only obtained following direct sequence analysis and requires additional labeling reactions and often the use of C-stretch flanking primers. By gaining knowledge of length variants prior to dye-terminator sequencing, the analyst can setup the sequencing analyses accordingly. This significantly increases turnaround of challenging mtDNA samples and alleviates repetition of result analysis.

The current study has validated the utility of HPLC to purify and quantify mtDNA amplicons in a single step. As an additional step in streamlining mtDNA

analysis, HPLC also yields length characteristics of mtDNA amplicons prior to dye-terminator sequencing. The quality of mtDNA sequences obtained in this study show that HPLC purification and quantification outperforms current standards in mtDNA sample processing.

§ 3 - 5 Summary

High-Performance Liquid Chromatography (HPLC) using a DNASep® column was evaluated as a means of quantifying PCR product yield and simultaneously purifying target amplicons for downstream applications such as DNA sequencing. The approach is based on the inherent ability of HPLC to fractionate by size, quantify and physically recover macromolecules. Using a commercial six fragment DNA quantification standard, a standard curve was generated for both UV and fluorescence detection, followed by recovery, quantification and sequence analysis of a variety of casework and reference-type samples. Samples purified by HPLC were compared to commonly used forensic purification methods (*i.e.,* ExoSAP-IT® and Spin column) and yielded improved sequencing results. Samples purified and quantified by HPLC yielded significantly improved QV and QV20+ scores over data reported in the general forensic literature. Determination of length-heteroplasmy within mtDNA amplicons yielded additional and important pre-sequencing data not seen by other methods. The current study demonstrates that HPLC has tremendous utility in

streamlining purification and quantification of target amplicons for downstream applications and in the same step determining length-heteroplasmy of mtDNA amplicons.

Chapter 4: Peak Height Reproducibility as an Indicator of Mitochondrial DNA Sequence Quality for Forensic Analyses

§ 4 - 1 Introduction

Dye-terminator sequencing is one of the most widely used methods in modern molecular genetics and medical diagnostics. This approach has also been established as the technology of choice for typing hypervariable regions 1 and 2 (HV1 and HV2) of the control region of the human mitochondrial DNA (mtDNA) genome by forensic laboratories. In this context, mtDNA sequencing has been used to analyze a diversity of evidentiary materials including hair shafts, saliva and bone even in cases where the biological source material has undergone significant decomposition or environmental insult (Hagelberg and Clegg, 1991; Hagelberg *et al.*, 1991; Holland *et al.*, 1993; Allen *et al.*, 1994; Ivanov *et al.*, 1996; Allen *et al.*, 1998).

Positive, negative and reagent blank DNA amplification/labeling controls are typically included in the standard operating procedures of forensic mtDNA laboratories for quality assurance/quality control purposes. These serve to demonstrate that the reagents used in this process are functional and free of extraneous contaminating DNA. The resulting DNA sequence data are then assessed primarily on the basis of basecalling quality scores and the watchful eye

of trained forensic analysts. A variety of sequence vicissitudes, however, can often compromise what would otherwise be the straight forward process of basecalling. These include, differential mobilities of DNA fragments due to chemical differences among the dye-terminators, differential dye-terminator incorporation efficiencies (Parker *et al.*, 1996), excessive "noise" from unincorporated dye-terminators, sequence compressions - especially in area of GC-richness (Sanger and Coulson, 1975), and a general loss of peak-to-peak signal resolution with poor signal-to-noise ratios toward the end of long electropherograms.

Basecalling software programs processes DNA sequence electropherogram files and assign a base quality score to each call using the equation $QV=-10\log_{10}(Pe)$ where *Pe* is the probability of an error in a base call (Biosystems, 2003). Based on an examination of peak spacing, the ratio of the largest uncalled to the smallest called peak and the degree of peak resolution expected in a given window, it is typically possible to distinguish between errors and correct base-calls. The "KB" Basecaller from Applied Biosystems (Foster City, CA) employs such an algorithm.

While base quality scores indicate the confidence with which individual base calls have been made relative to theoretical optimum, they do not necessarily serve as a measure of the extent to which the quality of a sequencing

reaction deviates from what would be seen for an actual reference sample. It is thus conceivable that base quality scores alone might not readily reflect subtle differences in the reproducibility of sequencing reactions. Such differences can be significant in a forensic context when comparing sequence data generated from different laboratories, different DNA extracts, different input DNA quantities or different labeling kits. Similarly, base quality scores do not necessarily compensate for reproducible anomalies (*e.g.*, deviations from the theoretically optimal base spacing) that are commonly seen in sequence electropherograms.

In cycle sequencing reactions, the efficiency with which dye-labeled terminators are incorporated can be highly variable between any two positions reflecting both the chemical differences between the dye-terminators and the influence of sequence context (Khan *et al.*, 1999). The resulting electropherograms, therefore, are typically characterized by a pattern of peaks that are often significantly uneven in their heights relative to each other. Such variability can have a negative impact on basecalling confidence with existing basecalling algorithms. The ability to predict relative differences in peak heights, however, could be used to improve sequence accuracy. Moreover, if the pattern of peak height variability were highly reproducible, the pattern itself could be employed as a parameter for assessing the quality of a given sequencing reaction relative to an actual reference standard. This has important implications for

laboratory validation and quality assurance efforts since it would enable analysts to automatically spot check a sequence to determine whether the global "electrophoretic pattern" is within an acceptable range of sample-to-sample variability for a specific amplicon and the instrumentation in a specific laboratory.

Quantifying the reproducibility of an "electrophoretic pattern" may also facilitate the analysis of mixed samples including heteroplasmic as well as situational mixtures of more than one individual. This is based on the reasonable expectation that after accounting for spectral differences in dye terminator fluorochromes, relative differences in peak heights are fundamentally a function of the underlying difference in the quantity of terminated DNA molecules. Thus, any significant change in the relative height of a given peak should reflect a change in the underlying quantity of DNA at that position. Some researchers have attempted to quantify the level of heteroplasmy by comparing the relative heights of overlapping electrophoretic peaks (Sekiguchi *et al.*, 2003). In the absence of data on peak height reproducibility, however, such measures are only rough approximations. In an effort to achieve greater quantitative accuracy, some researchers have relied on alternative approaches such as quantitative real-time PCR (Bai and Wong, 2004), pyrosequencing (White *et al.*, 2005) or endpoint assays that employ densitometry (Tengan *et al.*, 1997). These

approaches, however, consume additional sample while often being laborious or requiring instrumentation not typically found in a forensic laboratory.

The first objective of the current study, therefore, was to assess the quantitative reproducibility of electrophoretic peak heights for the HV1 and HV2 regions of human mtDNA as a function of DNA input quantity, base location and sequencing chemistry. The second objective was to determine whether a quantitative pattern of relative peak height differences across a high-quality reference electropherogram could be used to accurately assess the quality of DNA sequencing results from subsequent sequencing reactions.

§ 4 - 2 Materials and Methods

§ 4 - 2.1 Mitochondrial DNA Extraction and Purification

This research was conducted in compliance with U.S. Federal Policy for the Protection of Human Subjects (56 FR 28003). Samples were collected from subjects who were unrelated and had provided informed consent to participate in the study. Mitochondrial DNA was extracted from buccal swabs using the EZ1 DNA tissue kit on the Qiagen BioRobot EZ1 DNA extraction robot (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. Briefly, whole swabs were immersed in 190µL of Qiagen's proprietary "G2 Buffer" and 10µL of proteinase K solution (600mAU/mL), vortexed for 10 sec. and incubated at 56°C for 15 min. The digested supernatant was transferred to a sterile 2mL sample

tube and loaded onto the BioRobot EZ1 where samples were automatically extracted using paramagnetic beads and eluted into 200µL DNA free water. Validated primer pairs and PCR amplification conditions (Wilson *et al.*, 1995; Budowle *et al.*, 2000) were used to amplify four forensically relevant regions of the human mitochondrial control region (*i.e.,* HV1A, HV1B, HV2A and HV2B amplified by primer sets, A1/B2, A2/B1, C1/D2 and C2/D1, respectively) except that Ampli*Taq* GOLD® DNA polymerase (Applied Biosystems) was used and supplemented with high-fidelity *Pfu* DNA polymerase (Stratagene, La Jolla, CA) at a 9:1 ratio.

Amplification reactions were purified by denaturing high-performance liquid chromatography (Huber *et al.*, 1993; Kuklin *et al.*, 1997) on a WAVE® 3500HT Nucleic Acid Fragment Analysis System (Transgenomic Inc., Omaha, NE). Briefly, unpurified PCR products were bound to a DNASep® analytical column at 50°C in the presence of 0.1M triethylammonium acetate (TEAA) pH 7.0 as an ion pairing reagent. Target PCR amplicons were selectively eluted using a linear gradient of increasing acetonitrile produced by differential mixing of buffer A (0.1M TEAA) and buffer B (0.1M TEAA: 25% ACN). The optimal gradients employed a 56% to 65% buffer B increase in 3.5 minutes for HV1A, HV2A and HV2B and a 55% to 64% buffer B increase in 3.5 minutes for HV1B. All samples were eluted at 0.9ml/min flow rate and detected by UV at 260nm. Eluted peaks were captured using an automated fraction collector; dried by vacuum

centrifugation (30 minutes at 50°C, followed by a 20-minute cool down to ambient temperature); resuspended in ddH_2O; quantified by 260/280nm UV spectrophotometry and stored frozen until dye-terminator labeling.

§ 4 - 2.2 Mitochondrial DNA Sequencing

DNA sequencing reactions were prepared for each of four control region amplicons. Use of the eight aforementioned PCR primers for labeling allowed each amplicon to be sequenced in both the forward and reverse directions. Reactions prepared using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) used five different DNA input quantities (0.1ng, 0.2ng, 0.4ng, 0.8ng and 1.6ng) while those prepared using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) used three different DNA input quantities (0.4ng, 0.8ng and 1.6ng). For all samples and conditions tested, five independently-labeled replicate 10µl reactions were prepared in accordance with the manufacturer's protocol. Appropriate positive and negative controls were carried through the entire sample handling process to monitor for contamination and reagent integrity.

Dye-terminated products were purified by Performa® DTR V3 96-well short plate purification columns (Edge Biosystems, Gaithersburg, MD) per the manufacturer's protocol and resolved on a PRISM® 310 Genetic Analyzer (Applied Biosystems) using POP-6™ polymer and 47cm x 50µm capillaries

(Applied Biosystems). The raw electrophoretic traces were analyzed using the KB Basecaller together with the dye set mobility files indicated for the labeling kit used. The resulting sequence data were displayed using the Sequencher™ DNA analysis software (Gene Codes Corp, Ann Arbor, MI).

Quantitative data on peak heights for statistical analyses of electrophoretic patterns were obtained by first analyzing raw electrophoretic data using the KB Basecaller which is part of the PRISM® 310 Sequencing Analysis Software version 5.1 (Applied Biosystems). The resulting .scf output file was then ported to the CEQ™ 8000 Genetic Analysis System version 8.0 software package (Beckman-Coulter, Fullerton, CA) to allow output of the electrophoretic peak height data for each channel and time point as a .txt file. Text files were then opened in Microsoft Excel for subsequent statistical analyses. The height of each peak in relative fluorescence units (RFU) was compared across the five replicates for each amplicon, sequencing primer, input DNA quantity, and sequencing chemistry tested.

§ 4 - 3 Results

The reproducibility of electrophoretic traces across the mtDNA HV1 and HV2 region was evaluated. A total of five different DNA template input quantities (0.1ng - 1.6ng); encompassing a range frequently encountered by forensic practitioners were used. For these analyses, each of four forensically-relevant

mtDNA amplicons was sequenced in both the forward and reverse directions. The resulting eight dye-terminator labeling reactions were independently replicated five times each for statistical purposes.

Figure 4.1 presents an example of peak height and statistical variability data (RFU ± SD) for nucleotide positions 140 - 180 of HV2A at labeling reaction DNA input quantities of 0.4ng, 0.8ng and 1.6ng. Across these DNA input quantities, the heights of individual electrophoretic peaks and, therefore, the overall "pattern of relative peak height differences" was highly reproducible. This is exemplified by the consistently low peaks for 147C and 164C versus the consistently high peaks for 143G and 160A. Although the data presented in figure 4.1 represent a small portion of the HV2A amplicon, they are consistent with the results that have been obtained for the entire HV1 and HV2 region. The sequencing traces obtained using lower DNA input quantities (0.1ng and 0.2ng) were similarly consistent (Appendix B).

§ 4 - 3.1 Impact of DNA input quantity on peak height reproducibility

Shown in Table 4.1 are peak height variability data for each of the eight labeling reactions used to sequence the HV1 and HV2 regions. These values reflect the variability in peak height averaged across all the peaks in the amplicon sequenced. Also shown is the combined peak height variability average of all sequencing reactions at each of five DNA input quantities. In order to

compensate for the often significant differences in peak heights between any two positions, variability data were expressed as a percentage of peak height (*i.e.*,. Coefficient of variation = SD/mean x 100%).

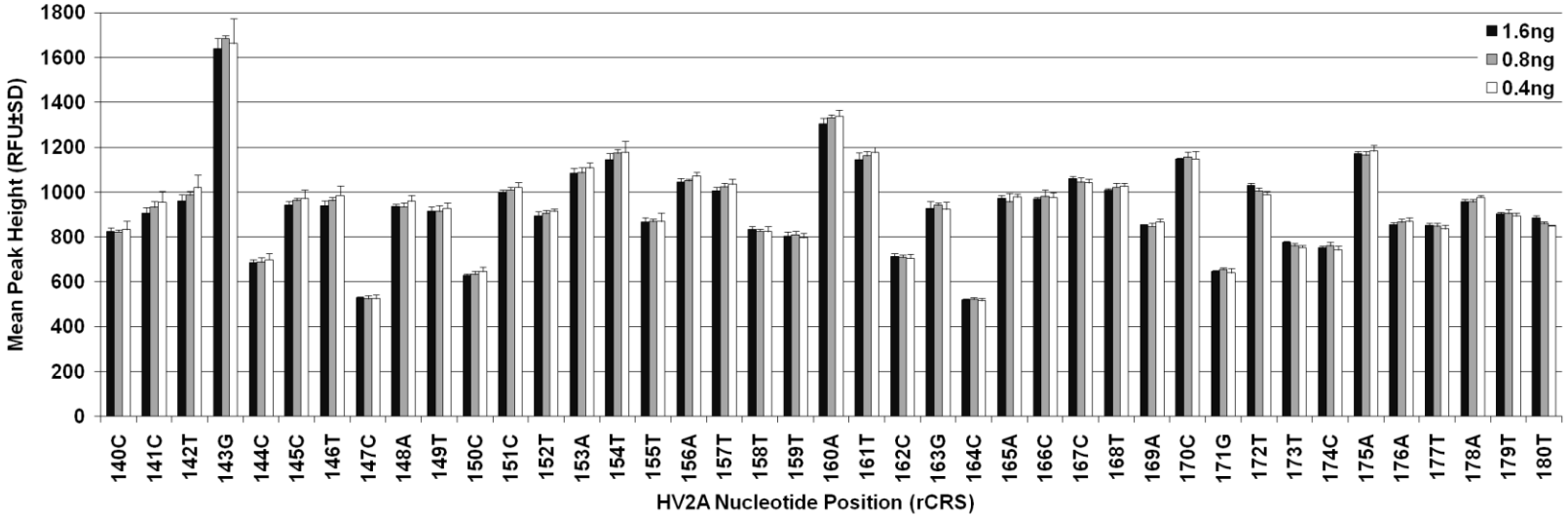


Figure 4.1: Reproducibility of electrophoretic peak heights for positions 140 to 180 of the mtDNA HV2A amplicon. The data represent the mean peak heights ± standard deviations from five independent labeling reactions for each of the three DNA input quantities. Although there are significant differences in mean peak heights between different nucleotide positions, the mean peak heights for each individual nucleotide position are highly consistent across the range of DNA input quantities shown.

Table 4.1: Total variability (coefficient of variation with standard deviation normalized to individual peak heights) associated with direct sequence electropherograms for each of the four forensically relevant mtDNA amplicons which encompass HV1 and HV2.

DNA Quantity	A1	B2	A2	B1	C1	D2	C2	D1	Average
0.1ng	6.0%[a]	8.6%[a]	8.5%	3.6%[a]	5.3%[a]	3.8%	5.4%	10.0%[a]	**6.4%**
0.2ng	2.7%[a]	6.5%[a]	4.4%	2.6%[a]	4.3%[a]	5.7%	5.1%	7.5%[a]	**4.9%**
0.4ng	2.7%	5.7%	3.5%	2.2%	5.6%	1.4%	8.6%	8.3%	**4.8%**
0.8ng	2.2%[b]	8.6%	4.6%	3.2%	2.3%	1.4%	3.4%	6.0%	**4.0%**
1.6ng	7.2%[b,c]	7.5%[b]	2.6%[b]	1.5%[b]	2.2%[b,c]	3.5%[b]	4.3%	8.0%[b]	**4.6%**

[a] elevated noise at the beginning of the electropherogram

[b] signal deterioration at the end of the electropherogram

[c] signal saturation across the electropherogram

Using the BigDye® Terminator v1.1 Cycle Sequencing Kit, 0.8ng of input DNA yielded the lowest overall variability (±4.0%) across all amplicons. The lowest input DNA quantity tested (0.1ng) had the highest average peak height variability at ±6.4%. At lower input DNA quantities (0.1ng and 0.2ng), there was a marked decrease in the signal-to-noise ratio particularly at the beginning of an electropherogram. Conversely, electropherograms generated using 1.6ng of input DNA were frequently characterized by fluorescence signal saturation, especially of G and T peaks and/or a marked degradation in peak resolution near the end of the trace.

§ 4 - 3.2 Impact of positional location and sequence context on peak height reproducibility

To determine whether a base's positional location within an amplicon has an influence on electropherogram reproducibility, peak height variability was compared across the length of each sequenced amplicon at five DNA input quantities. Although the average variability across an amplicon (Table 4.1) ranged from ±1.4% (HV2A sequenced using the D2 primer at 0.4 - 0.8ng input DNA) to ±10.0% (HV2B sequenced using the D1 primer at 0.1ng input DNA), variability was found to be very position dependent. As illustrated by an analysis of HV1A amplicon sequenced using the B2 primer (Figure 4.2), peak height variability is typically greatest immediately adjacent to the primer binding site. Variability decreases steadily towards the center of the amplicon; normally

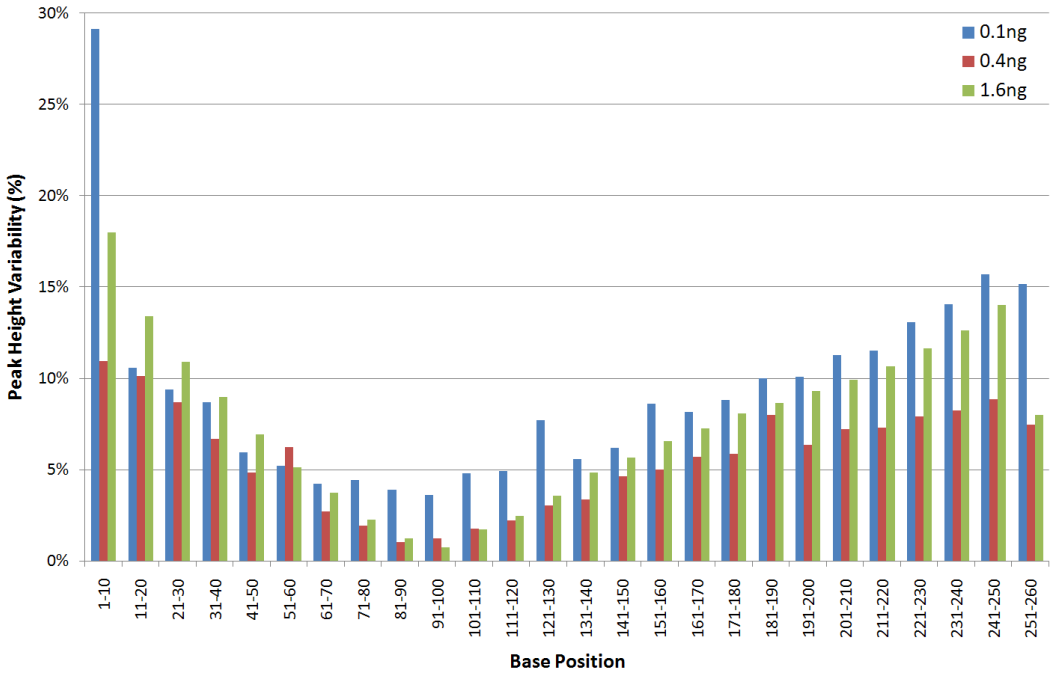


Figure 4.2: Variability of electrophoretic peak heights across the HV1A amplicon sequenced using the B2 primer. To compensate for relative differences in peak heights, variability data (averaged for groups of ten bases) are expressed as a percentage of peak height. Values are based on five independent labeling reactions for each of the three DNA input quantities. Variability which is greatest adjacent to the primer binding site decreases towards the center of the amplicon and then rises toward the end of the labeled product. This pattern is more pronounced at input DNA quantities of 0.1ng and 1.6ng.

reaching a minimum 85-114bp downstream of the primer binding site. Variability then rises steadily as one approaches the end of the labeled product. This phenomenon is generally more pronounced at either low (0.1 - 0.2ng) or high (1.6ng) input DNA quantities (Appendix B).

Nearest neighbor interactions are important to the thermodynamic stability of base pairing in nucleic acids. To determine whether this has a significant impact the efficiency of nucleotide incorporation during dye-terminator labeling, peak height variability was examined as a function of the preceding base for all HV1 and HV2 amplicons at all five input DNA quantities. Analysis of the resulting data by two-way non-parametric Kruskal-Wallis test showed no significant influence or interaction on peak height variability as a result of the preceding base at any DNA input quantity ($H=0.0913$, $df=8$, $P=0.999$).

§ 4 - 3.3 Impact of sequencing chemistry on peak height reproducibility

To determine whether peak height reproducibility is influenced by sequencing chemistry, two DNA input quantities (0.8ng and 1.6ng) were tested across the four labeling reactions used to sequence the HV1 region. Peak height variability averaged across an amplicon was found to be more variable with the BigDye® Terminator v3.1 than the v1.1 Cycle Sequencing Kit. At 0.8ng of input DNA, peak height variabilities for HV1 sequencing reactions ranged from 2.5% -

16.4% with the v3.1 chemistry versus 1.6% - 9.9% with the v1.1 chemistry. Similar results were obtained at 1.6ng of input DNA where the average peak height variabilities for HV1 sequencing reactions ranged from 2.6% - 21.5% with the v3.1 chemistry versus 1.4% - 7.5% with the v1.1 chemistry. In addition to being more consistently reproducible, the v1.1 chemistry also yielded higher quality sequence data at lower DNA input quantities; provided sequence information immediately adjacent to the primer binding site and; more clearly resolved peaks throughout the electropherogram (Figure 4.3).

§ 4 - 3.4 Evaluating sequence data relative to a "reference electropherogram"

Given the overall reproducibility of "electrophoretic patterns", it should be possible to use relative differences in the heights of adjoining peaks to assess how closely a "questioned electropherogram" conforms to a "reference electropherogram" and thereby to identify electrophoretic irregularities of potential interest in a questioned sequence. As an initial test of this approach, the electropherogram for HV1A (0.8ng of input DNA; A1 primer; the v1.1 chemistry) was selected as a reference sequence. Using a prior moving average equation:

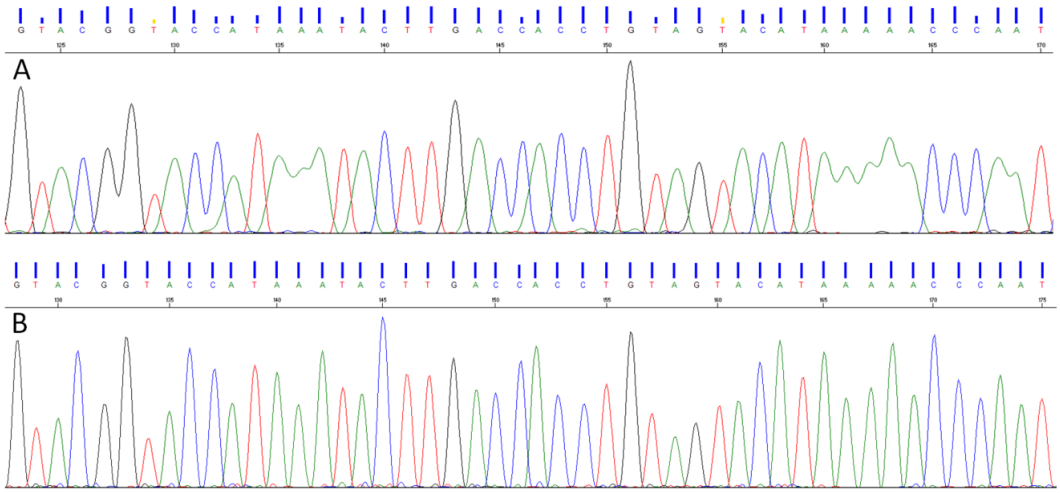


Figure 4.3: Examples of DNA sequence electropherograms obtained using BigDye® Cycle Sequencing chemistries. (3A) The v3.1 chemistry results in electropherograms characterized by lower quality scores and reduced resolution between adjoining peaks especially in regions of homopolymeric sequence. (3B) The v1.1 chemistry results in electropherograms characterized by higher quality scores and optimal resolution between adjoining peaks.

where “Q” and “R” are peak heights (RFU) in the questioned and reference samples, respectively, “P” is the predicted peak height and “n” is the base position in the electropherogram it is possible to generate a “predicted peak height pattern” for any questioned electropherogram based on a reference electropherogram. By comparing the “expected heights” to the actual peak heights in the questioned sample, it is possible to assess how closely the questioned electrophoretic pattern corresponds to the reference pattern.

Figure 4.4 illustrates the use of this approach for comparative pattern analysis between the HV1A reference sequence mentioned above and the electropherogram for the HV1A region of a questioned sample. Deviations from the expected peak heights are readily indicated by the presence of “pattern perturbation spikes” in the comparison plot. In figure 4.4, there are a total of five spikes which represent deviations from the expected “reference pattern.” Three of these spikes (16086, 16129 and the compound spike from 16182 - 16186) are due to the fact that the questioned sample was a two-component (80:20 ratio) DNA mixture. The spikes reflect changes in the height of the major component peak where the underlying minor component differs in primary sequence (*i.e.*, mixed based positions: 16086C/T, 16129A/G, 16182A/C, 16183A/C and 16184T/C). The spike at 16089 reflects deviations from expected peak heights due to the proximal influence of the T→C base substitution at position 16086 in the minor component of this DNA mixture. Similarly, the spike at position 16185

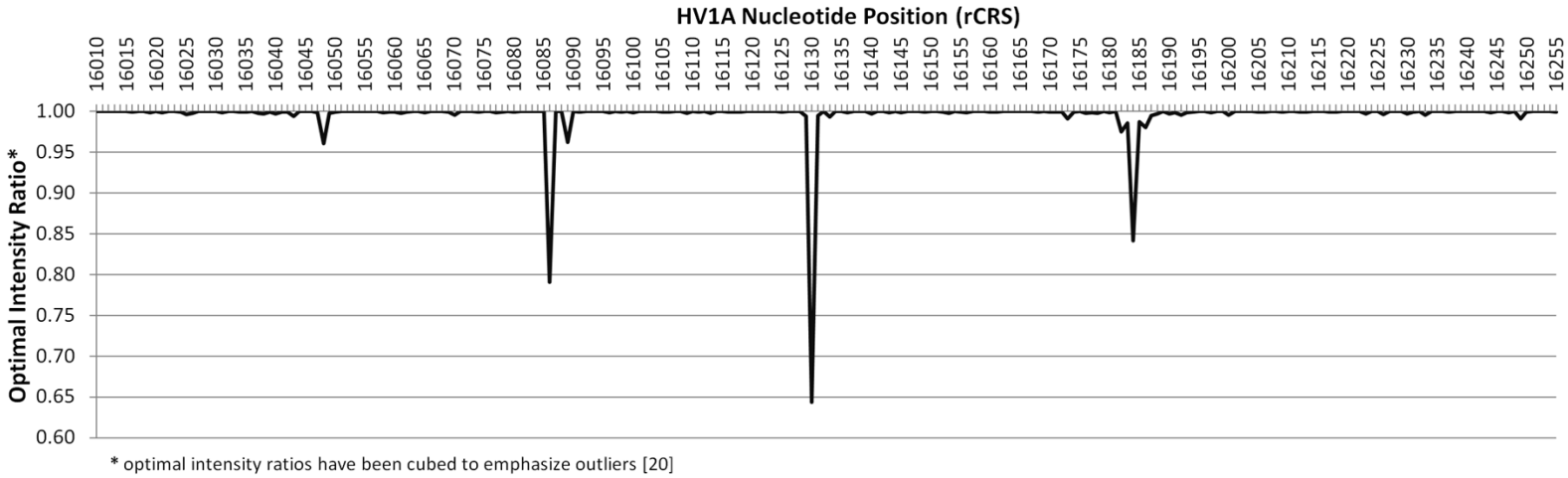


Figure 4.4: Electrophoretic pattern comparison plot between a reference and a questioned HV1A sample. Spikes represent deviations in peak height of the questioned electropherogram from expected values. Here, these deviations reflect: mixed based positions in the questioned sample (16086C/T, 16129A/G, 16182A/C, 16183A/C and 16184T/C); the influence of a base substitution on proximal bases (*e.g.*, the influence of the 16086T→C substitution on the peak at 16089); and a suspected anomaly in the basecalling software (position 16048). (*optimal intensity ratios have been cubed to emphasize outliers (Cramer, D., 1997).

- 16186 results from the proximal influence of the neighboring substitutions at positions 16182 - 16184. The spike at position 16048 cannot be traced to the direct/proximal influence of a base substitution and thus represents an “atypical perturbation spike”. Because they are unattributable to base substitutions, atypical spikes represent potential sequence electropherogram anomalies of particular interest. These phenomena will be addressed in the following sections.

In a broader test of comparative electrophoretic pattern analyses, forty non-identical questioned samples (ten each for HV1A, HV1B, HV2A and HV2B) were compared to the cognate reference electropherograms pattern. Table 4.2 shows the number of pattern perturbation spikes for each amplicon. These spikes were categorized as either “base substitution”, “proximal substitution” or “atypical” spikes. For all amplicons, base substitutions present in the questioned amplicons accounted, directly or through proximal effect, for 289 of 321 (90.0%) pattern perturbation spikes. Atypical spikes were less common, accounting for 32 of 321 (10.0%) spikes.

§ 4 - 3.5 Impact of base substitution on proximal peak heights

To assess the impact of base substitution on the overall “pattern” of relative peak heights, sequencing traces representing each of four mtDNA amplicons were compared. Each amplicon was sequenced in both the forward and reverse directions at five different DNA input quantities (0.1ng - 1.6ng).

Table 4.2: Total number and causal distribution of peak perturbation spikes detected in pairwise analyses of 40 non-identical samples relative to the cognate reference sample for each amplicon and labeling primer.

Amplicon	Primer	Base Substitution	Proximal Substitution	Atypical Spikes	Total Spikes
HV1A	**A1**	2	2	0	4
HV1A	**A1**	6	0	1	7
HV1A	**A1**	4	5	1	10
HV1A	**A1**	0	0	0	0
HV1A	**A1**	5	2	0	7
HV1A	**B2**	0	5	1	6
HV1A	**B2**	3	4	0	7
HV1A	**B2**	2	5	0	7
HV1A	**B2**	4	7	0	11
HV1A	**B2**	9	5	0	14
HV1B	**A2**	5	8	2	15
HV1B	**A2**	5	0	3	8
HV1B	**A2**	6	3	2	11
HV1B	**A2**	2	3	5	10
HV1B	**A2**	3	0	1	4
HV1B	**B1**	11	3	0	14
HV1B	**B1**	5	2	0	7
HV1B	**B1**	8	3	1	12
HV1B	**B1**	1	4	0	5
HV1B	**B1**	9	9	2	20
HV2A	**C1**	3	5	0	8
HV2A	**C1**	3	0	0	3
HV2A	**C1**	2	2	0	4
HV2A	**C1**	3	3	1	7
HV2A	**C1**	5	4	0	9
HV2A	**D2**	0	0	0	0
HV2A	**D2**	3	8	0	11
HV2A	**D2**	5	5	0	10
HV2A	**D2**	1	4	1	6
HV2A	**D2**	4	2	0	6
HV2B	**C2**	5	2	1	8
HV2B	**C2**	2	2	0	4
HV2B	**C2**	2	12	2	16
HV2B	**C2**	1	1	0	2
HV2B	**C2**	1	3	0	4
HV2B	**D1**	0	2	0	2
HV2B	**D1**	0	4	4	8
HV2B	**D1**	8	2	0	10
HV2B	**D1**	6	5	1	12
HV2B	**D1**	6	3	3	12

A base substitution may influence the peak heights of surrounding bases. Based on analyses of 150 base substitution sites, the height of peaks within a range of -2 to +5 bases of the substitution site are most typically affected. This is not to suggest, however, that every peak within this window will necessarily be affected nor that such proximal effects cannot occur beyond it. Figure 4.5 illustrates this for a T→C base substitution at position 16086. In addition to the expected change in channel in which the dye terminator fluorochrome is detected and height of the electrophoretic peak at the substitution point, the heights of the nearby peaks also showed a reproducible change in height. The largest changes were seen at 16084G, 16088T and 16089G. The heights of 16084G and 16088T decreased while that of 16089G increased following the base substitution at 16086. The intervening peak heights for 16085C and 16087A, by contrast were unchanged.

§ 4 - 3.6 Other anomalous impacts on peak heights

Although the majority of pattern perturbation spikes are attributable to base substitutions, atypical spikes appear to have a different etiology. Among atypical spikes in the current study, 75.0% could be traced to poor signal quality typically at the beginning or end of a sequencing electropherogram. The remaining 25.0% of atypical spikes, however, were present in regions of high quality signal. In an effort to elucidate the underlying cause of the remaining

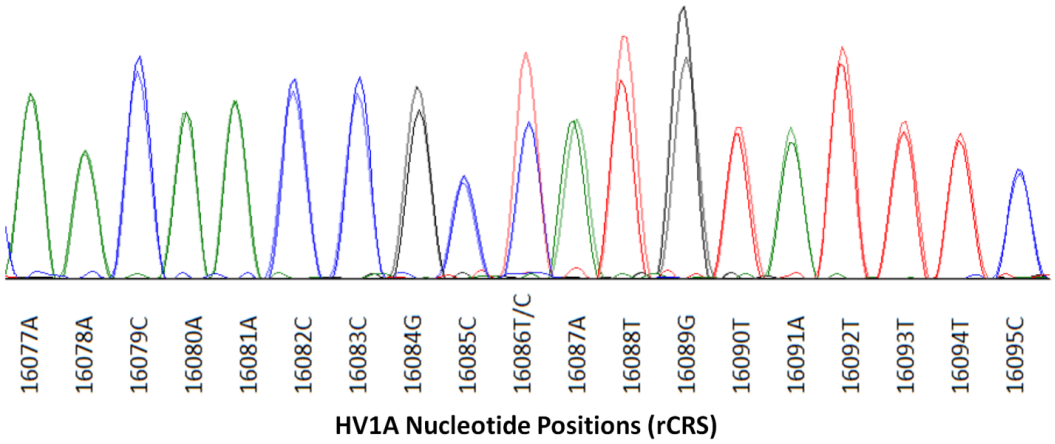


Figure 4.5: Superimposed electropherograms for a region of HV1A sequenced using the A1 primer illustrated the influence of a 16086T→C base substitution on the heights of proximal peaks. The largest changes involved decreases in peak heights at 16084G and 16088T and an increase at 16089G. This proximal effect did not impact the heights of the intervening peaks at 16085C or 16087A.

"atypical spikes", raw and analyzed electropherograms were compared for these anomalous positions which were present in all four mtDNA amplicons.

Figure 4.6 shows the analyzed electrophoretic traces for five replicate sequencing reactions of part of the HV1A amplicon sequenced with the B2 primer. While there is almost complete consistency in the height for each peak across these independent reactions, the peak corresponding to 16182T has a significantly reduced height in the fifth replicate sample. This results in an atypical spike when compared to a reference sequence. Analyses of other atypical spikes in regions of good signal quality revealed a similar pattern (*e.g.*, positions 16182 in HV1A; 16267 and 16284 in HV1B; and 158 in HV2A). In each case, the anomalous peak was nearly always the second base of a 3 - 5 base homopolymeric A- or C-stretch; reduced in height relative to the reference pattern; and did not appear to have any influence on the heights of neighboring bases.

§ 4 - 4 Discussion

Electrophoretic peak heights reflect the underlying quantity of DNA molecules terminated at a given position during the labeling process. The current study provides an evaluation of the reproducibility of peak heights in sequence electropherograms for human mtDNA amplicons commonly employed for forensic analyses. A thorough understanding of specific factors that may impact

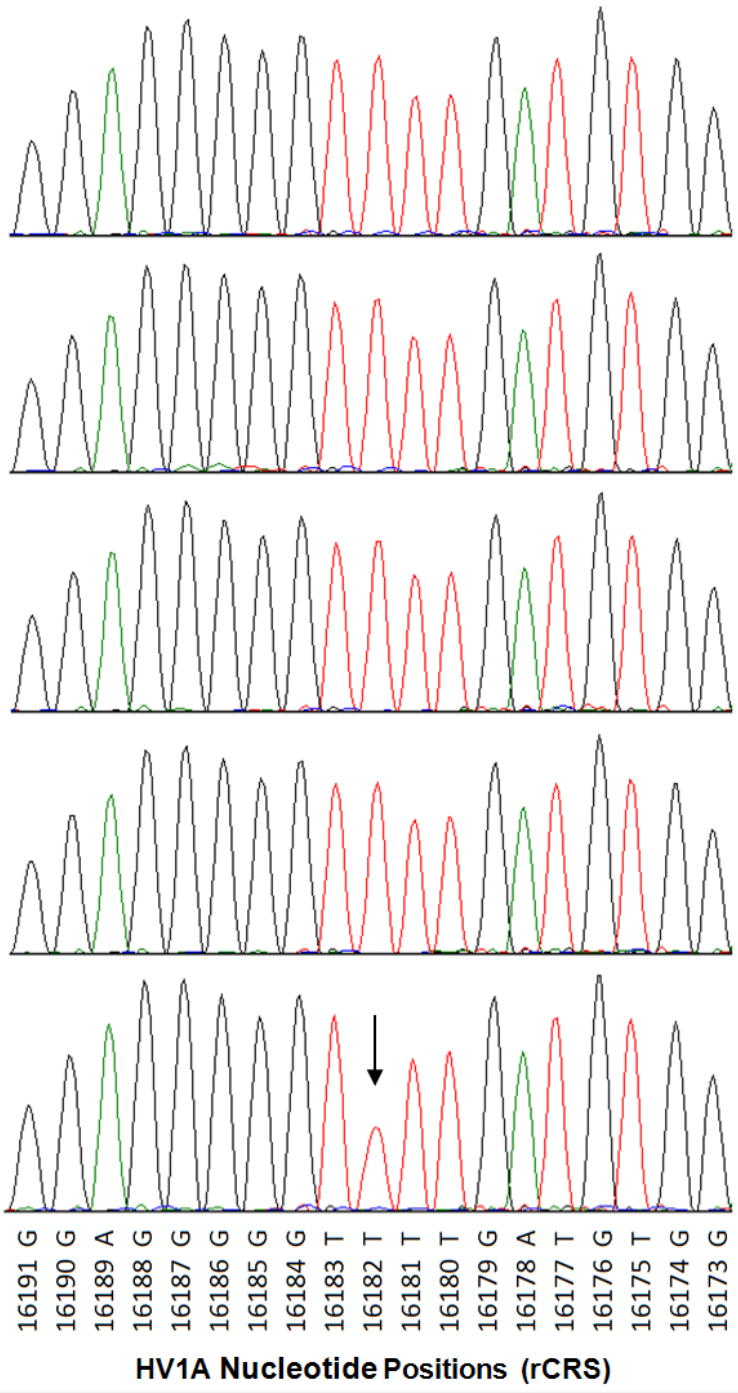


Figure 4.6: Replicates of five sequencing reactions from HV1A sequenced with the B2 primer and the overall peak height consistency for each peak across multiple independent reactions. Note that the 16182T peak (arrow) of the last replicate is significantly lower than the corresponding peak in the preceding four replicates.

the reproducibility of a given electrophoretic trace can make it possible to rapidly identify genuine sequence irregularities thereby facilitating more efficient and accurate interpretation of mtDNA sequence data by lab analysts.

DNA template quantities have a significant influence on peak height reproducibility and sequence quality. Across all four forensically-validated mtDNA amplicons, 0.4 - 0.8ng of input DNA yielded the lowest average peak height variability. Electropherograms generated using these optimal input quantities showed minimal noise associated with unincorporated dyes at the beginning of the sequence and minimal signal deterioration at the end of the sequence. Elevated peak height variability and lower signal-to-noise ratios were frequently seen with low DNA input (0.1 - 0.2ng) while signal saturation at high input (1.6ng) produced “phantom peaks” due to spectral pull up.

Deconvolution of saturated electrophoretic peaks, which is required for basecalling, results in both an underestimation of a peak’s true height and greater peak height variability. Signal saturation also produces pull-up of the overlapping dye spectra. This artifact remains following application of the dye matrix and typically results in the appearance of false “phantom peaks” immediately before or after the saturated peak. Figure 4.7 illustrates this in a comparison of a 43bp stretch of HV1A sequence data for two DNA input quantities (1.6ng and 0.8ng). Signal saturation seen in the raw data for the 1.6ng

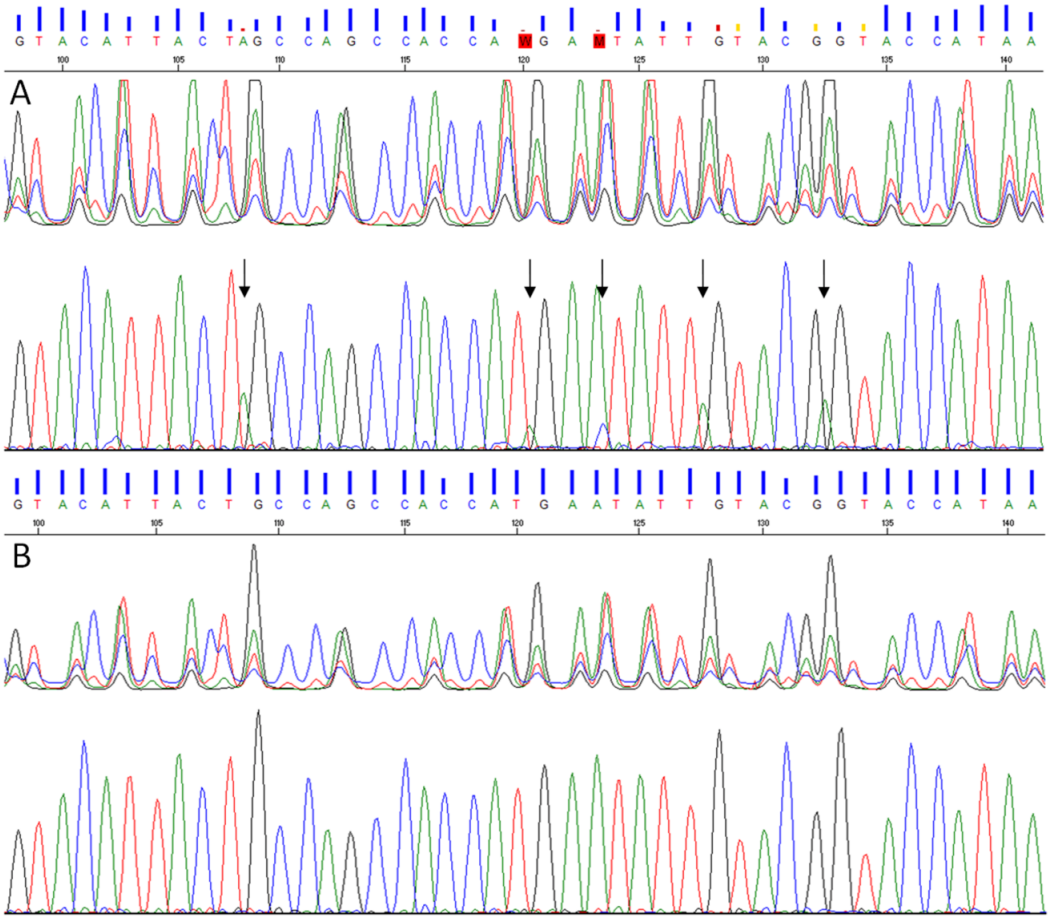


Figure 4.7: Comparison of raw and analyzed electrophoretic sequence data for HV1A sequenced using the A1 primer at two DNA input quantities. (7A) Use of 1.6ng of template DNA in a dye-terminator labeling reaction was associated with: signal saturation of the raw data; phantom peaks produced by spectral pull-up in the analyzed data and; frequent low basecall quality scores. (7B) Use of 0.8ng of template DNA in a labeling reaction eliminated these anomalies in the analyzed data and resulted in basecall quality scores that were consistently high.

reaction (Figure 4.7A) resulted in the appearance of five clearly defined phantom peaks in the analyzed data – three of which were erroneously called by the sequencing software. In the absence of signal saturation, these were eliminated as illustrated by the analyzed data for the 0.8ng reaction (Figure 4.7B). This underscores the importance of accurate template quantification prior to dye-terminator labeling.

Given the sequence dependent folding characteristics of a DNA molecule, the context specificity of a base might be expected to influence the reproducibility of dye-terminator incorporation. Although small differences in peak height reproducibility were seen as a function of the preceding base, these differences did not approach statistical significance. The degree of electropherogram reproducibility was, however, found to vary as a function of location within an amplicon. Peak height variability was elevated both in the region immediately downstream of the labeling primer and at the end of the amplicon leaving the most reproducible peak heights clustered toward the center of the amplicon. Although this was seen at all DNA input quantities, use of optimal input DNA quantities (0.4 - 0.8ng) minimized the phenomenon.

Two BigDye® Terminator Cycle Sequencing chemistries (v1.1 and v3.1) are commercially available for forensic applications. Although most forensic laboratories employ the v1.1 chemistry for mtDNA sequencing, the v3.1

chemistry has also been used for forensic casework (Cruz *et al.*, 2004). On the basis of both reproducibility and peak quality, the v1.1 kit demonstrated superior performance. Quantitatively, electropherograms produced using the v1.1 kit were nearly twice as reproducible. Qualitatively, interpretable sequence reads typically began almost immediately after the primer and the resolution between peaks was greater – especially in homopolymeric regions (Figure 4.3). More critically for forensic laboratories, the v1.1 kit yields high quality sequence information at half the DNA input quantity required with the v3.1 kit. This is an advantage in cases where only minute quantities of DNA are recovered (*e.g.*, degraded biological material).

Given the reproducibility of sequencing electropherograms and having characterized the impact of DNA input quantity, positional location and sequencing chemistry, it may be possible to develop algorithms capable of assessing sequence quality relative to a reference electropherogram. In the current study, reproducible differences in the heights of neighboring electrophoretic peaks were used as a basis for comparison. When the height of an electrophoretic peak was either significantly higher or lower than what was expected based on the electropherogram from a corresponding reference sample, that peak resulted in the formation of a “pattern perturbation spike” as described previously. A number of factors, many of potential interest to a forensic analyst, can cause an electropherogram to deviate from the expected

reference pattern. These include base substitutions, mixed base positions and changes in the heights of peaks in the vicinity of a base substitution (*i.e.*, "proximal affects").

The approach may also facilitate the detection of subtle sequence anomalies that are not evident from base quality scores. In the current study, for example, the occurrence of some "atypical spikes" revealed the presence of anomalies that were not linked to base substitutions, mixed base positions or regions of elevated peak height variability. Figure 4.8 highlights two bases (16267C and 16284A) which were found to be independently associated with atypical spikes. In figure 4.8A, the height of the 16267C peak is consistent with expectations based on the reference electrophoretic pattern while 16284A is abnormally low. Conversely, in figure 4.8B, this relationship is reversed such that 16267C is abnormally low while the 16284A peak is consistent with the reference electrophoretic pattern. These anomalously low peaks, however, showed no corresponding indication of an anomalously low fluorescence signal in the raw data. Rather, the raw data appears indistinguishable between "normal" traces and those in which an atypical spike was seen. Furthermore, there does not appear to be an increase in noise or an obvious change in peak morphology that could account for this anomaly. Re-analysis of the same data set using a difference basecaller (*i.e.,* ABI basecaller) eliminated the anomaly, suggesting a software problem. In consultation with software engineers at Applied

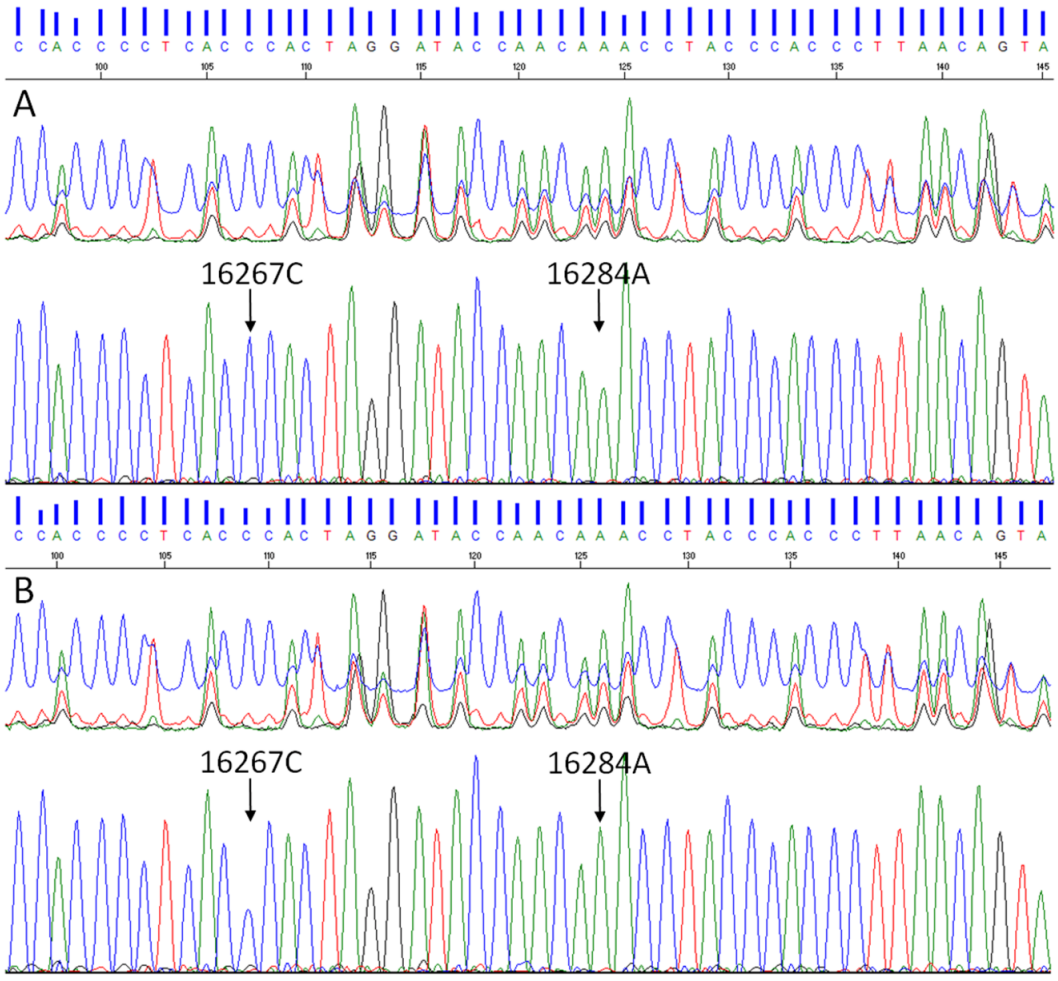


Figure 4.8: Illustration of the independent occurrence of two peak height anomalies within the same region of two of five replicates sequencing reactions. (8A) A low peak height anomaly is present at position 16267C while the peak height for 16284A is within expected values. (8B) A low peak height anomaly is present at position 16284A while the peak height for 16267C is within expected values. Although the anomalies are clearly evident in the analyzed data, comparison of the corresponding peaks in the raw data traces show no significant difference in peak height suggesting that the anomaly is due to a factor other than a reduction in fluorescence signal.

Biosystems, the consensus opinion is that the anomaly is likely the result of an unidentified bug within the KB Basecaller (Applied Biosystems, personal communication). The potential for this to adversely impact basecalling accuracy has not been determined. The fact that its occurrence can be detected through pattern analysis may assist analysts by focusing their attention on potentially problematic sequence data.

The algorithm employed for the current study represents only one possible approach to the quantitative comparison of electrophoretic patterns. The results presented, while encouraging should not be interpreted at indicating that alternative algorithms might not provide superior detection of regions of sequence data that deviate from that of a reference pattern. The development and validation of an optimal algorithm was beyond the scope of the current study but represents an important focus for future research.

Comparative analysis of electrophoretic patterns may have important potential as an added computational tool for forensic analysts working with mtDNA. First, the entire process can be readily automated and as a result may help to more readily direct an analyst's attention to regions of sequence data worthy of heightened scrutiny. By using a known sample as the reference electropherogram, it could enable an analyst to more quickly and confidently include or exclude a questioned sample. From a laboratory QA/QC perspective

this approach may be useful as a rapid and reliable means of monitoring sequencing kit integrity and instrument performance based on pattern concordance with a laboratory reference standard.

§ 4 - 5 Conclusion

DNA sequencing analysis is among the most comprehensive genetic analysis tools currently used in molecular biology. For forensic applications, it remains the gold standard for mtDNA haplotyping. To date, however, the assessment of DNA sequence quality has relied almost exclusively on the experienced eye of forensic analysts and the use of base quality scores. The results of the current study have evaluated the reproducibility of sequencing electropherograms and quantitatively characterized the impact of DNA input quantity, peak location, sequencing chemistry and base substitution on peak height variability. This study has potential benefits for forensic practitioners in that the data presented may facilitate the development of computational approaches to aid in the laborious process of reviewing mtDNA sequence electropherograms. This can speed the turnaround of mtDNA samples while allowing for greater confidence in base calls and quicker disposition of forensic cases. Ongoing studies seek to develop and ultimately validate statistically grounded algorithms in conjunction with software for automated data analysis.

§ 4 - 6 Summary

The review of dye-terminator sequencing electropherograms is central to forensic mtDNA haplotyping. The assessment of DNA sequence quality relies heavily on the experienced eye of the forensic analyst and the use of basecall quality scores. The current study sought to assess the quantitative reproducibility of electrophoretic peak height patterns for forensically relevant regions of human mtDNA. The impact of DNA input quantity, base location and sequencing chemistry on peak height variability and the potential utility of comparative electrophoretic pattern analysis was evaluated. DNA template quantities used in dye terminator labeling reactions were found to significantly influence sequence quality and reproducibility. DNA input quantities of ≤0.2ng resulted in elevated peak height variability and lower signal-to-noise ratios while input ≥1.6ng produced signal saturation associated with false peaks. Peak height variability was position dependent across all amplicons being lowest in the center and highest adjacent to the primer binding site. With regard to sequencing chemistries, the BigDye® Terminator v1.1 Cycle Sequencing chemistry demonstrated superior performance on the basis of peak height reproducibility, electropherogram quality and optimal DNA input quantity.

The reproducibility of "electrophoretic patterns" makes it possible to use relative differences in the heights of adjoining peaks to assess how closely a "questioned electropherogram" conforms to a "reference electropherogram"

and thereby to identify electrophoretic irregularities of potential interest in a questioned sequence. A comparative analysis of forty, non-identical samples representing the HV1A, HV1B, HV2A and HV2B amplicons accurately identified deviations from reference electrophoretic patterns which resulted directly or indirectly from base substitutions, the presence of mixed base positions in situational mixtures and a possible anomaly in the basecalling software. Comparative analysis of electrophoretic patterns may have important potential as computational tool for forensic analysts. Such an approach may help analysts to more rapidly identify sequence irregularities; thereby facilitate more efficient and accurate interpretation of mtDNA sequence data.

Chapter 5: Quantitative Determination of Mixed Nucleotide Populations in Mitochondrial DNA Sequencing

§ 5 - 1 Introduction

There has been significant discussion regarding mtDNA and the importance of characterizing heteroplasmy and situational mixtures in the forensic and other scientific literature (Ivanov *et al.*, 1996; van Den Bosch *et al.*, 2000; Melton *et al.*, 2005). Much of the discussion has focused on the basic detection of heteroplasmy; the potential value of being able to quantitatively assess heteroplasmic ratios. It has been proposed that the availability of quantitative information might aid human identification (Gill *et al.*, 1994), disease diagnostics (Harrigan *et al.*, 1998; Nurpeisov *et al.*, 2003) and the ability to determine the individual mtDNA haplotypes of the contributors to a mtDNA mixture (Danielson *et al.*, 2005). Dye-terminator sequencing has through the years been considered to be the most accurate method of DNA sequence analysis in molecular genetics and molecular diagnostics. As new terminator chemistries have been developed, consistency in peak height and reliability of base calls have become a major focus of scrutiny in dye-terminator sequencing. Individual peak heights across a sequencing electropherogram are highly variable relative to one another. This is due to differences in the incorporation rates of individual dye-terminator nucleotides at a given base position and the spectral

properties of their associated fluorochromes. For any specific base position, however, peak heights are highly consistent across multiple sequencing reactions (Chapter 4).

In dye-terminator sequencing, the appearance of a heterozygous loci or a mixture of two or more DNA molecules is characterized by one or more overlapping electrophoretic peaks. This typically results in ambiguous basecalls at mixed sites. In the case of a length variant, a completely unreadable sequencing electropherogram is produced starting at the point where the two variants fall out of register with each other. The quantitative ratio of the contributors to a mixture should be reflected by differences in the peak heights at mixed-base positions. Since peak height is governed in part by the relative incorporation efficiencies of individual nucleotides, the ratio of one peak to another at a mixed-base position is not a direct reflection of the relative DNA quantity contribution of two or more contributors to a mixture. (Danielson *et al.*, 2007). As a result, mixed-base positions in sequencing electropherograms from forensic casework samples are generally omitted from the reported haplotype analysis. This exclusion of data decreases the power of discrimination of the analyzed sample.

In spite of these limitations, some researchers have attempted to use peak height data to follow the spread of drug resistant mutations associated with human immunodeficiency virus type 1 (HIV-1) (Harrigan *et al.*, 1998; Nurpeisov *et*

al., 2003) and the spread of mutations in hepatitis B virus polymerase (Olivero *et al.*, 2006). This approach relies on a comparison of relative peak heights for the variants of interest at mixed-base positions from samples taken at different time points. An increase in nucleotide peak height associated with the resistant/disease strain along with a corresponding decrease in peak height associated with the wild type nucleotide suggests an increase in the prevalence of the resistant/disease strain between the samples being assayed.

Additional methods for the detection of these mutations have been developed. These include line probe assays (Stuyver *et al.*, 1997) (Innogenetics, Gent, Belgium) and detection by real-time PCR (Affigene, Sangtec Molecular Diagnostics AB, Bromma, Sweden). Although these approaches work well when dealing with known genetic markers, they require the design of new nucleotide specific probes whenever a new sequence variant is discovered. An added concern is that line probe assays are associated with a high level of false negative results (Servais *et al.*, 2001). Real-time PCR assays are accurate and highly reproducible but the detection of mutations at multiple different sites necessitates the design and costly optimization of numerous assays. In a forensic context, these methods can consume a significant amount of often precious evidentiary material.

The ability to extract quantitative information on DNA mixtures from the direct analysis of peak height ratios at mixed-base positions in sequencing electropherograms would make it possible to circumvent these limitations. Such an approach would allow for analysis of known and novel polymorphisms regardless of location within an amplicon. Furthermore, quantitation analyses could be conducted without the need for any additional sample manipulation. Finally, sequencing analyses require significantly lower template quantities than a single line probe assay and can be completed at significantly lower cost than either of the aforementioned method.

Although dye-terminator sequencing has, in general, been very successful for accurate DNA haplotyping of single source samples, the interpretation of DNA heteroplasmy and the analysis of mixtures of two or more individuals have proven to be significantly more difficult. Due to context specificity of nucleotide incorporation efficiencies, the reliable determination of relative DNA quantity associated with each component of a mixture has until now been unattainable. The ability to accurately interpret DNA mixtures would have significant benefits for basic research, disease diagnostics and forensic investigations involving mtDNA or other sequence-based analyses such as those based on biallelic markers. The objective of the current study was, therefore, aimed at determining the extent to which a change in the relative quantities of input DNA for two

amplicons correlated with a change in ratio between the resulting electrophoretic peak heights across a range of mixed-base positions.

§ 5 - 2 Materials and Methods

This research was conducted in compliance with U.S. Federal Policy for the Protection of Human Subjects (56 FR 28003). Individual buccal swabs were collected from 45 unrelated subjects of diverse ethnicity who had previously provided informed consent to participate in the study. All samples were stored at -20°C until DNA extraction.

§ 5 - 2.1 Mitochondrial DNA Extraction and Purification

Mitochondrial DNA was extracted from buccal swabs using the EZ1 DNA tissue kit on the Qiagen BioRobot EZ1 (Qiagen Inc., Valencia, CA) according to the manufacturer's recommended protocol. Four forensically relevant regions of the human mitochondrial control region (*i.e.*, HV1A, HV1B, HV2A and HV2B) were amplified in 50µL reactions, using 50pmol each of forensically-validated PCR primers (Wilson *et al.*, 1995; LaBerge *et al.*, 2003); 2.25U Ampli*Taq* GOLD® DNA polymerase (Applied Biosystems, Foster City, CA) supplemented with 0.25U *Pfu* DNA polymerase (Stratagene, La Jolla, CA); Ampli*Taq* GOLD® Buffer (Applied Biosystems); 10nmol of each dNTP (Stratagene) and 10µL of an approximately 10pg/µL human DNA extract. Amplifications were performed on a GeneAmp® 9700 thermocycler (Applied Biosystems) with an initial denaturation at 95°C for

10 minutes, followed by 32 cycles of 95°C for 20 seconds, 60°C for 30 seconds and 72°C for 45 seconds. The final extension at 72°C was for 15 minutes. PCR amplicons were purified and quantified at 50°C on a WAVE® 3500HT DNA Fragment Analysis System (Transgenomic Inc., Omaha, NE) containing a DNAsep® analytical column packed with alkylated poly(styrenedivinylbenzene) resin (Huber *et al.*, 1993) using previously established methods (Chapter 3).

§ 5 - 2.2 Mitochondrial DNA Sequencing

Mixture templates were prepared at thirteen stepped quantity ratios ranging from 99:1-1:99 (*i.e.*, 99:1, 95:5, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 5:95, 1:99) at 0.4ng/µl. Sequencing reactions were prepared in triplicate 10µl reactions, using the previously mentioned forensically-validated primers. For each labeling reaction, 0.8ng of input DNA was used in order to obtain optimal peak height consistency as described previously (Chapter 4). Samples were extended using the BigDye® v1.1 Dye Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's protocol. Dye-terminated products were purified using Performa® DTR V3 96-well Short Plate Kit (Edge BioSystems, Gaithersburg, MD) according to the manufacturer's protocol and resolved on the PRISM® 310 Genetic Analyzer (Applied Biosystems). Appropriate positive and negative controls were carried through the entire sample handling process to minimize cross contamination and to monitor amplification efficiency.

Sequence data was analyzed with Sequencing Analysis Software v.5.1.1 (Applied Biosystems). The resulting .scf output file was then ported to the CEQ™ 8000 Genetic Analysis System version 8.0 software package (Beckman-Coulter, Fullerton, CA) to allow output of the electrophoretic peak height data as a .txt file. Text files were then opened in Microsoft Excel for statistical analyses or displayed using the Sequencher™ v4.2 DNA analysis software (Gene Codes Corp, Ann Arbor, MI).

§ 5 - 3 Results and Discussion

§ 5 - 3.1 Reproducibility of Peak-Height Ratios

Representatives of each of the four forensically validated mtDNA amplicons (*i.e.*, HV1A, HV1B, HV2A and HV2B) that collectively encompassed 100 variant sites across these regions were originally examined. During the course of theses analyses, it was noted that the relationship between relative quantities of input DNA and the ratio of the resulting electrophoretic peak heights at mixed-base positions was not identical when sequenced in the forward and reverse directions. In discussions with forensic practitioners, a more rigorous approach was taken to demonstrate the relationship between relative quantities of input DNA and the ratio of electrophoretic peak heights at mixed-base positions by including an additional 102 mixed-base positions in the reverse sequencing direction (*i.e.*, a total of 202 mixed-base positions, Table 5.1). In addition, 5

Table 5.1: Sequence polymorphisms in the HV1 and HV2 regions assayed in two-component mixtures at 13 different DNA quantity ratios. Nucleotide differences are noted as deviations from the revised Cambridge Reference Sequence.

	HV1	HV2
Transitions	16051 G/A 16051 T/C 16069 C/T 16069 G/A 16086 A/G 16086 C/T 16093 G/A 16093 T/C 16104 C/T 16104 G/A 16111 C/T 16111 G/A 16114 A/G 16114 T/C 16126 A/G 16126 C/T 16129 A/G 16129 C/T 16136 T/C 16144 C/T 16144 G/A 16145 C/T 16148 A/G 16148 T/C 16163 C/T 16163 G/A 16172 A/G 16172 C/T 16173 A/G 16173 T/C 16179 C/T 16179 G/A 16184 G/A 16184 T/C 16186 A/G 16186 T/C 16187 C/T 16187 G/A 16189 A/G 16189 C/T 16192 A/G 16192 T/C 16193 A/G 16193 T/C 16209 A/G 16209 C/T 16217 C/T 16217 G/A 16222 G/A 16222 T/C 16223 A/G 16223 C/T 16224 C/T 16224 G/A 16239 A/G 16239 T/C 16241 C/T 16241 G/A 16243 A/G 16244 A/G 16244 T/C 16249 A/G 16249 T/C 16256 A/G 16256 T/C 16261 G/A 16261 T/C 16266 G/A 16270 A/G 16270 T/C 16274 C/T 16274 G/A 16278 A/G 16278 C/T 16286 C/T 16286 G/A 16291 A/G 16291 T/C 16293 C/T 16293 G/A 16294 A/G 16294 C/T 16295 A/G 16295 T/C 16296 C/T 16296 G/A 16298 G/A 16298 T/C 16299 C/T 16299 G/A 16304 A/G 16304 C/T 16309 A/G 16309 C/T 16311 A/G 16311 C/T 16320 C/T 16320 G/A 16325 A/G 16325 T/C 16327 C/T 16327 G/A 16343 C/T 16343 G/A 16352 C/T 16352 G/A 16353 A/G 16353 T/C 16355 C/T 16355 G/A 16360 C/T 16360 G/A 16362 A/G 16362 T/C 16390 G/A	64 A/G 64 T/C 72 C/T 72 G/A 73 A/G 73 C/T 89 A/G 89 T/C 92 C/T 92 G/A 93 A/G 93 C/T 143 A/G 143 T/C 146 A/G 146 T/C 150 C/T 150 G/A 151 G/A 151 T/C 152 A/G 152 C/T 153 A/G 153 T/C 182 G/A 182 T/C 185 A/G 185 C/T 188 A/G 188 C/T 189 C/T 189 G/A 192 C/T 192 G/A 194 A/G 194 T/C 195 A/G 195 C/T 196 C/T 196 G/A 198 C/T 198 G/A 199 C/T 199 G/A 200 A/G 200 T/C 204 C/T 204 G/A 207 A/G 207 T/C 217 C/T 217 G/A 222 A/G 222 C/T 225 C/T 225 G/A 226 A/G 226 T/C 228 A/G 228 C/T 239 A/G 247 A/G 247 C/T 248 T/C 250 C/T 250 G/A 285 T/C 295 C/T 297 A/G 297 T/C 309 T/C 310 C/T 316 C/T 316 G/A 340 A/G 340 T/C
Transversions	16126 G/C 16129 C/G 16145 T/A 16182 A/C 16183 A/C 16265 A/C 16265 G/T 16286 C/G 16290 C/G	186 A/C 186 G/T 189 C/A 189 T/G 249 A/T 250 A/T 280 C/G

mixtures assayed that represented different combinations of mixed-bases within the 202 positions included in the study.

Given the reproducibility of inter-individual differences in relative peak heights, it is reasonable to suggest that a change in the relative molar ratio of one component to another in a mixture will be reflected by a corresponding change in ratio of overlapping fluorescence signals at all mixed-base positions. The relationship between different molar ratios of two contributors to a mixture and the corresponding electrophoretic peak height ratios at mixed-base positions (182 T/C; 186 A/C; 189 C/A; 195 C/T; 198 T/C; 200 G/A) is illustrated in figure 5.1. As the molar ratio between the two contributors in this HV2A mtDNA mixture was varied in a stepwise manner, from a 90:10 to a 50:50 ratio of contributor 1 to contributor 2, there was a coordinated shift in the ratio of overlapping electrophoretic peaks at nucleotide positions where the two contributors differ from each other. In this example, six peaks representing nucleotides associated with contributor 1 (182 T; 186 A; 189 C; 195 C; 198 T; 200 G) display a coordinated decrease in fluorescence intensity relative to the peaks associated with contributor 2 (182 C; 186 C; 189 A; 195 T; 198 C; 200 A). Figure 5.2 illustrates the excellent peak height reproducibility within overlapping electrophoretic peaks. Shown are the peak heights and associated error bars for mixed-base position 182 T/C across nine of the DNA quantity ratios tested in triplicate. As expected, peak height consistencies, for this and the other 201

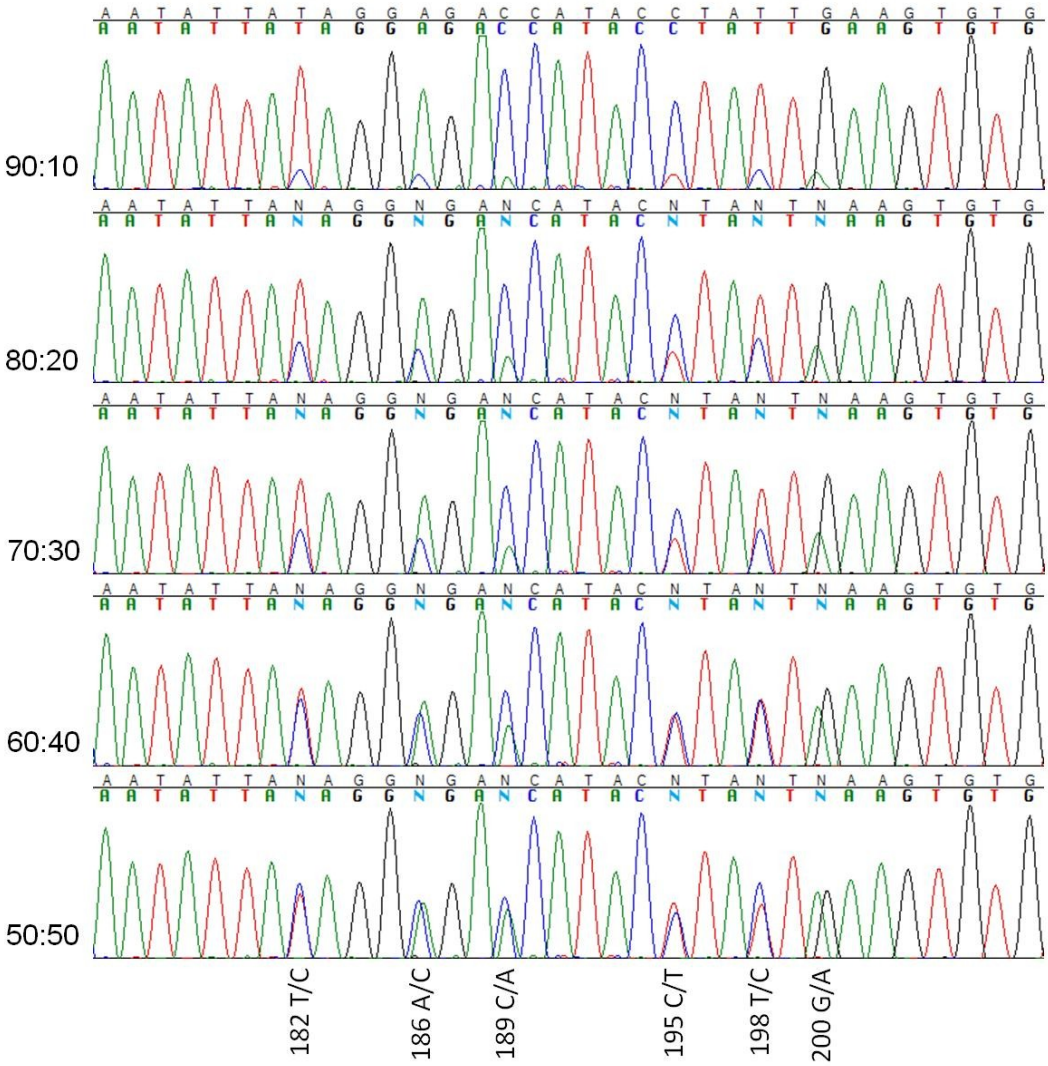


Figure 5.1: Fluorescence peak height ratios at six mixed-base positions as a function of stepwise changes in the quantity DNA input to the dye-terminator labeling reaction. Shown are mixture ratios ranging from a 90:10 mixture to a 50:50 mixture of two HV2 DNA amplicons.

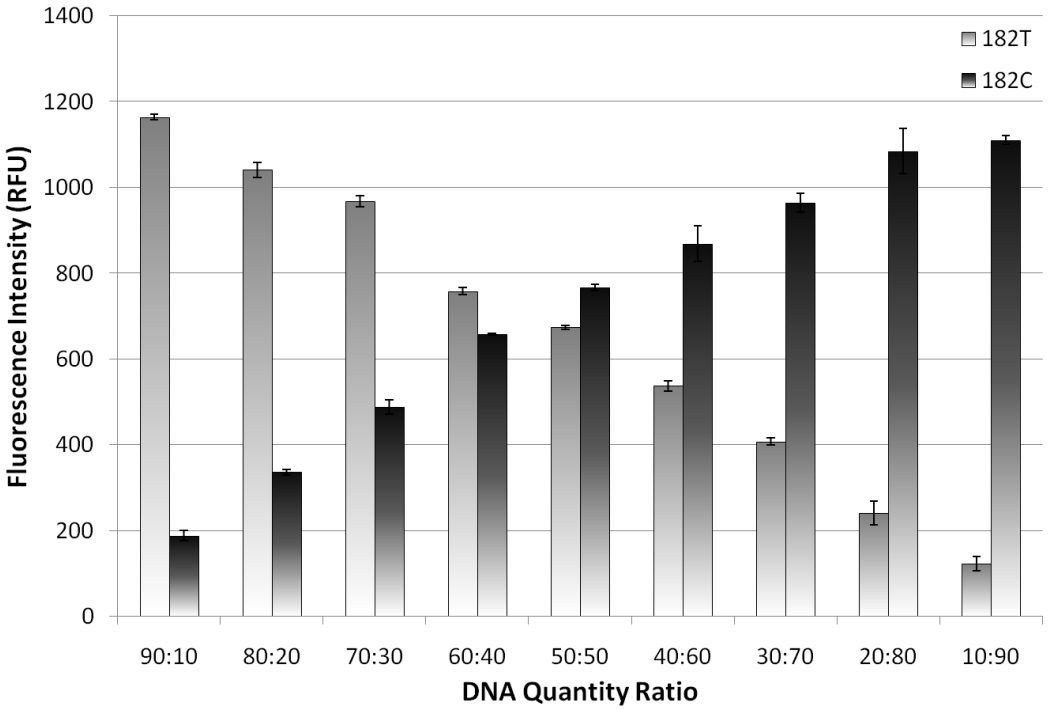


Figure 5.2: Peak height reproducibility at nt182 for three replicate labeling reactions at nine contributor DNA quantity ratios. Both components of the mixture show highly reproducible peak heights (as represented by the error bars) across all input quantity ratios tested.

mixed base positions included in this study, were highly consistent across three replicates. This confirms previously reported data on the global consistency of peak heights in sequencing electropherograms (Chapter 4). Furthermore, these results indicate that it should be possible to deconvolve the individual haplotypes of two-component mixtures.

§ 5 - 3.2 Correlation Between DNA Quantity and Fluorescence Intensity Ratios

The analyzed peak height data for 347 nucleotide mixtures revealed a highly significant correlation between changes in DNA input quantity ratio for a DNA mixture and changes in electophoretic peak height ratios. Consistent with the high degree of reproducibility that was observed previously for relative differences in electrophoretic peak heights (Chapter 4), a change in the molar ratio of one contributor to another was found to correlate nearly perfectly (R = 0.9998) with the change in relative fluorescence of overlapping peaks at mixed-base positions (Figure 5.3). This is not to suggest that all mixed-base positions are identical with respect to the relationship between a given peak height ratio and a given DNA quantity ratio. Instead, the relative peak height ratios observed at different nucleotide positions displayed highly reproducible context-specific peak height relationships.

This is best demonstrated by the highly reproducible differences seen in electrophoretic peak height ratios at a variety of nucleotide positions where

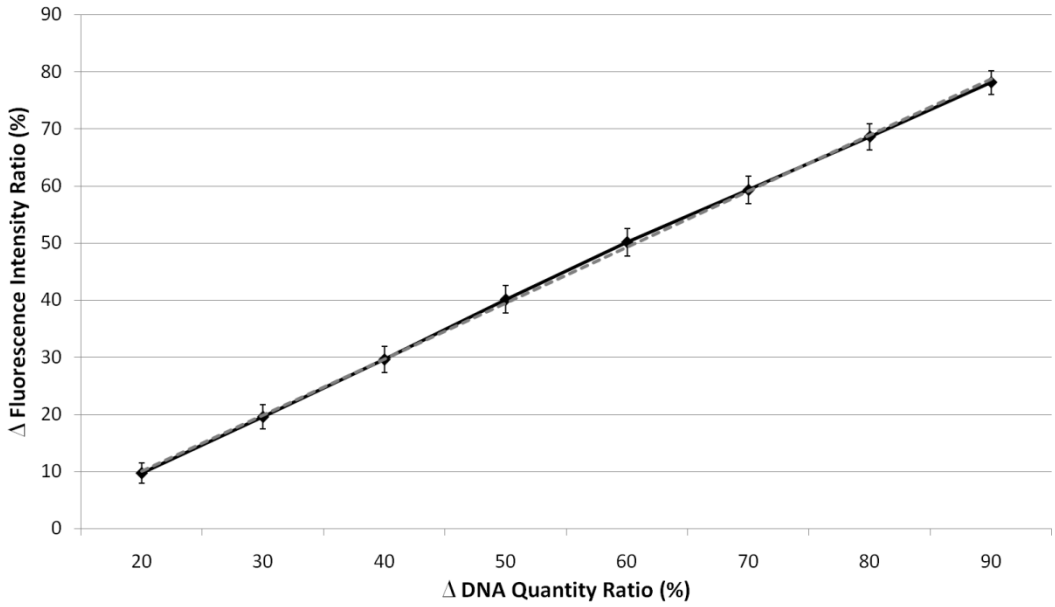


Figure 5.3: A graph illustrating the strong correlation (R = 0.9998) between a change in the molar ratio of DNA amplicons input to a dye-terminator labeling reaction and a change in peak height ratios at mixed-base positions. The plot consists of averages obtained from 74,952 data points (changes in peak height fluorescence). A total of 347 mixed nucleotide positions were assayed at 9 molar ratios of mixed mtDNA templates. The dashed line indicates the statistical line of best fit to the data.

equivalent DNA input quantities were assayed. Figure 5.4 illustrates the broad range of fluorescent peak height ratios observed at mixed-base positions in sequencing electropherograms when an equimolar mixture of two DNA amplicons is used for the labeling reaction. The displayed histogram shows the fluorescence contribution of the lower of the two peaks detected at each mixed-base position to the combined fluorescence signal of the overlapping peaks. Also shown is the frequency that each level of fluorescence was detected in the 347 assays of mixed-base positions that were analyzed. In the majority of cases (69.5%), the labeling of an equimolar DNA mixture was associated with a fluorescent peak height ratio that fell within ±10% of the ideal 50:50 ratio. There were a large number of cases that displayed significant discordance between an equimolar DNA input to the labeling reaction and the resulting fluorescence ratio at a mixed-base position. In the most extreme cases, the fluorescence ratio was strongly and reproducibly skewed to yield a 21:79 peak height ratio. Accordingly, for the 202 positions analyzed, one may reasonably assign a major and minor contributor on the basis of peak heights alone at mixed base positions providing that the major component peak exceeds 80% and the minor peak base call is less than 20% of the total fluorescence at a mixed base position.

§ 5 - 3.3 Impact of Peak Overlap on Fluorescence Intensity Ratio

Direct sequence analysis of mixed mtDNA templates yields overlapping peaks in the analyzed electropherogram at all positions where the templates

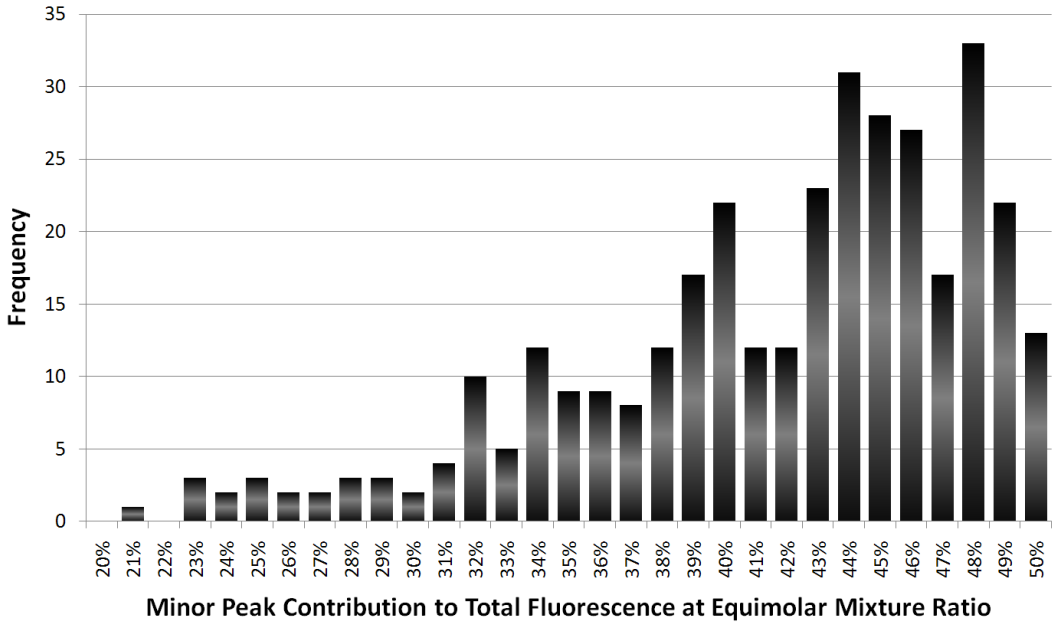


Figure 5.4: Frequency of minor peak contribution to total fluorescence at an equimolar (*i.e.*, 50:50) ratio of two contributors to a DNA mixture. In 69.2% of equimolar mixtures, the contribution of the minor peak to the total fluorescence at mixed base positions was within 10 percentage points of the idealized 50% value. In 30.8% of cases, however, the minor peak accounted for only 21% to 39% of total fluorescence at mixed base positions even though an equimolar mixture of amplicons was labeled. This phenomenon was highly reproducible across replicate reactions.

differ in primary sequence. In most cases, peaks representing these mixed base positions overlap completely (*i.e.*, peak maxima occur at the same sampling point). However, on occasion the minor peak at a mixed base position may be shifted significantly upstream or downstream of the major peak. When analyzed, this shift can result in an underestimation of the height of the minor component peak. At affected positions, this phenomena is highly consistent across all ranges of DNA input quantities (*e.g.*, position 200G/A in Figure 5.1) and has been noted to a varying degree at a number of different locations.

Figure 5.5A illustrates an extreme example of apparent minor component peak shifting for the 239A nucleotide of the minor component in a 60:40 mixture of 239G/A sequenced with the D2 primer. The sequence analysis algorithm places the 239A minor component peak between the 238T and 239G peaks creating an erroneous insert in the electrophoretic trace. The practical implications of this are significant. In a putative single-source DNA sample, such a peak shift could mistakenly be reported as an insertion. The risk of making such erroneous base calls underscores the importance of sequencing templates in both directions since this phenomenon has yet to be demonstrated in both directions. One possible explanation for this shift may be reproducible anomalies that exist in the spacing of some dye terminated chains. Figure 5.5B, illustrates this by showing that even in the absence of a 239A peak, the 239G peak is still shifted asymmetrically toward the 240T peak. It could be postulated that this

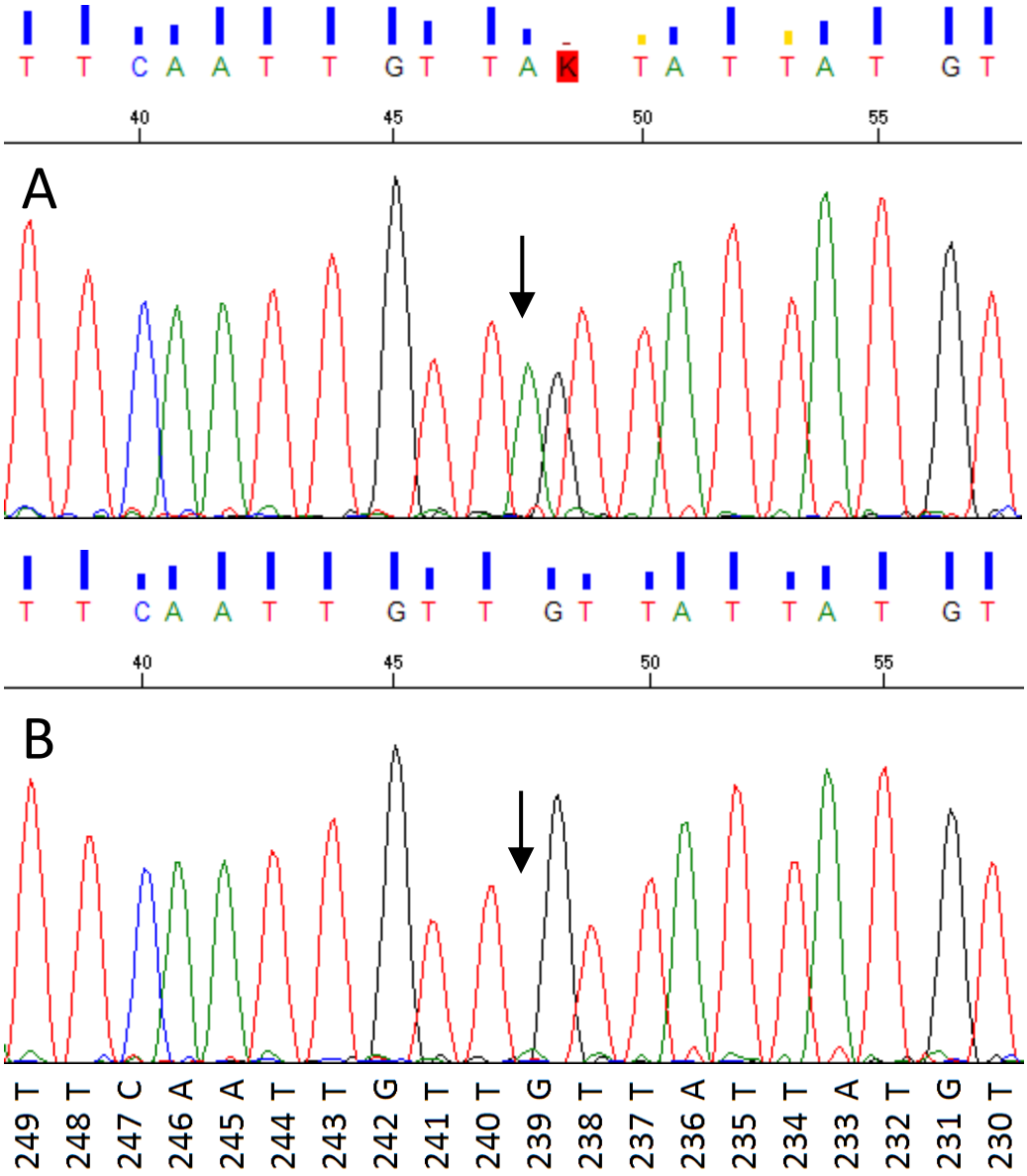


Figure 5.5: Positional shifting of a minor contributor peak relative to the major component peak in the HV2A amplicon sequenced with the D2 primer. **5A** illustrates a phantom insertion of 239A prior to the genuine 239G peak and an ambiguous base call (239K) a result of the inserted minor component peak. **5B** illustrates the peak spacing in the same region obtained with a single source sample. This demonstrates that even in a single source sample, 239G is positioned asymmetrically toward 240T. This could predispose mixtures at this position to phantom insertions.

asymmetry predisposes this position to such phantom insertions. Accordingly, analysts may be able to use base spacing in some cases as a potential indicator of whether a questioned insert is genuine or not.

§ 5 - 3.4 Minor contributor identification in low level mixtures

Direct sequence-based mixture determinations have generally been classified as any secondary/minor peak having a fluorescence intensity >10% of that of the major component. Although generally true, a number of factors can cause a mixed-base position to either under- or over-represent the relative quantity of DNA contributed by the secondary component. Analysis of 202 unique low-level mixed-base positions by direct DNA sequencing yielded a shift in fluorescence intensities for several tested positions. A total of 694 comparisons were performed at each of thirteen quantity ratios (*i.e.*, the aforementioned 99:1 through 1:99 range). At a quantity ratio of 20:80, 3.6% of minor component peaks yielded a fluorescence intensity ratio lower than the cutoff for calling mixed nucleotide positions (>10% major component peak height). At the 30:70 quantity ratio, however, no minor component peaks fell below the 10% mixture call threshold. As a result, some mixtures in which the minor contributor represents less than 30% of the total DNA quantity will be associated with selective peak dropout in the analyzed electrophoretic data even though the minor component itself accounts for >10% of the total input DNA quantity. In other words, some low level mixtures may yield a minor component

haplotype where one or more nucleotide substitutions are not detected. This has significant implications for haplotype determination of the minor component in mtDNA mixtures. In these cases, fractionation of cross hybridized PCR products by denaturing high performance liquid chromatography (DHPLC) can be employed to enrich for the minor contributor so as to avoid erroneous peak dropout (Danielson *et al.*, 2007).

These results beg the contrary question of how often a two-contributor mixture with a DNA input quantity ratio of 5:95 yields a fluorescence intensity ratio for the minor component that is greater than the 10% cutoff for mixed nucleotide detection. Out of 694 comparisons performed, 15.6% of minor components yielded a fluorescence intensity ratio greater than the 10% cutoff. While a small fraction of these can be traced to sequencing noise, the observed deviation between the DNA input quantity ratios and the corresponding fluorescence intensity ratio accounts for the majority of these cases. The obvious implication of this phenomenon is that a sample which may appear to be heteroplasmic at a single site, in reality, contains a low-level secondary component. Again, employing DHPLC fractionation can aid in detecting these types of low-level mixtures by enriching for the minor component. This will make it possible, in many cases, to determine the complete haplotype of the minor contributor. Alternatively, DHPLC could be used to purify away the minor component/contaminant to yield a clean sequence for the major contributor. In

all cases, knowledge of nucleotide incorporation rates associated with mixture determination is imperative.

§ 5 - 3.5 Additional Considerations

Previous published studies on relative peak height ratio determinations presented data from mixtures generated prior to PCR amplification (Harrigan *et al.*, 1998; Nurpeisov *et al.*, 2003). This assumes that both contributors to the mixture will amplify at equal efficiency regardless of template quantities present from each contributor. Where near equimolar quantities of each contributor are used it is probably reasonable to assume that the amplification efficiency of each contributor's amplicon will be approximately equivalent. This may not be as reasonable an assumption in cases where there is a great difference in the relative input quantities of two contributors. In fact, there may potentially be significant differences in amplification efficiency due to a relative scarcity of the minor component amplicon. This may skew the apparent contributor ratio towards the major contributor giving an inaccurate representation of the actual ratio that existed in the original mixture.

To demonstrate the extent to which this may present a point of concern, several two-contributor mixtures were prepared. Half of these mixtures were prepared before PCR amplification, and the remainder was prepared by mixing together stocks of DNA that had already been PCR amplified and quantified from

individual contributors. These mixtures were then dye-terminator labeled and the resulting relative peak height fluorescence ratios at 9 mixed base positions were analyzed. Figure 5.6 illustrates the results obtained at the mixed-base position 195C/T. The pre-amplification generated mixture shows a steeper slope across the mixture ratios assayed than was observed for the post-amplification generated mixture. The results obtained for the other 8 mixed base positions formed between these amplicons yielded similar results.

The results show that a bias in amplification efficiency exists toward the major component in non-equimolar ratio mixtures. Therefore, these results suggest that when determining peak height ratios in actual forensic mixture samples, these will nearly always tend to overestimate the major contributor to the mixture and underestimate the minor contributor.

§ 5 - 4 Concluding Remarks

The current study provides a comprehensive analysis of changes in relative peak heights at mixed base positions as a function of changes in relative DNA input quantities to dye-terminator labeling reactions. Previous studies have demonstrated the highly reproducible nature of peak height patterns in sequencing electropherograms (Chapter 4). Due to the sequence context dependence of electrophoretic peak height at individual base positions, peak height alone cannot generally be used to accurately determine the relative

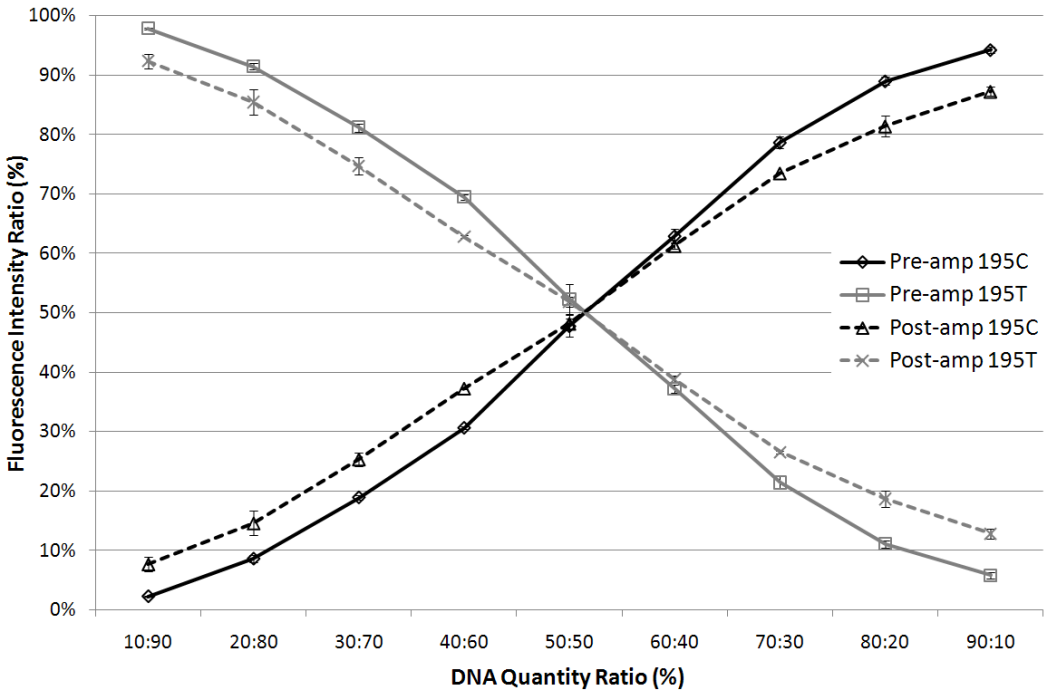


Figure 5.6: A range of DNA quantity ratios generated from pre- and post PCR amplification mixtures. Mixtures prepared prior to PCR amplification (solid lines) showing a steeper slope than mixtures prepared after PCR amplification of the contributors individually. This may be due to amplification bias against the less abundant of the target amplicon (*i.e.*, minor contributor) during PCR.

quantity of individual amplicons when present in a mixture. However, the strong statistical correlation between changes in the relative quantity of two amplicons and changes in the resulting ratio of overlapping fluorescent peaks at mixed base positions provides a potential means for accurately determining the relative quantities and thus the haplotypes of individual amplicons in a mixed sample. This is of particular utility to some efforts aimed at monitoring disease progression, the onset and spread of drug resistant strains of pathogens and mtDNA mixture analysis in forensics.

As an example of how changes in relative peak height ratios at mixed-base positions can be used to indentify individual haplotypes, consider the following. Given a change in the quantity of one contributor relative to another (*e.g.,* from a 90:10 ratio to a 70:30 ratio of contributor "1" to contributor "2"), a decrease will be observed in the peak height of all bases associated with amplicon "1" relative to amplicon "2". Conversely, an increase will be observed in the peak height of all bases associated with amplicon "2" relative to amplicon "1". By analyzing such shifts in relative peak heights at mixed-base positions, it should be possible to clearly and reproducibly determine the primary DNA sequence of (*e.g.*, the haplotype in the case of mtDNA) for each contributor to the mixture. Especially in cases where the minor contributor accounts for 20% or less of a mixed sample, accurate haplotype determination is often not possible due to potential peak dropout. In these cases, fractionation and enrichment of

individual contributors by DHPLC is indicated (Danielson *et al.*, 2007). It is expected that this approach will allow for the accurate determination of the linkage phase (*i.e.*, the DNA sequence) for individual contributors.

Using the mixture analysis strategy presented here, it should be possible to accurately deconvolve the DNA sequences of virtually all two-contributor mixtures. Moreover, the extensive body of information that is presented in this study in conjunction with the results of previous studies on peak height reproducibility as a function of DNA input quantity will facilitate the development of statistically supported interpretation guidelines for the analysis of mixed DNA samples.

§ 5 - 5 Summary

In dye-terminator sequencing, the appearance of a heterozygous locus or a mixture of two or more DNA molecules is characterized by overlapping electrophoretic peaks at all positions where the amplicons in a mixture differ in primary sequence. This results in ambiguous basecalls at mixed sites or unreadable sequencing electropherogram, in the case of indel-associated length variations between DNA molecules. The ability to accurately resolve DNA mixtures will have significant benefits for basic research, disease diagnostics and forensic investigations involving mtDNA analysis.

In mixed amplicon populations, the ratio between overlapping electrophoretic peaks at mixed base positions shifts as the molar ratio of the input DNA between individual contributors changes. If shown to be quantitatively reproducible, this phenomenon may provide a means of deconvolving the sequence electropherograms generated by sequencing DNA mixtures.

The objective of the current study, therefore, was to determine the extent to which a change in the relative quantities of input DNA from two amplicons correlated with a change in the ratio between overlapping electrophoretic peaks at mixed base positions. The current study presents peak height ratio data for 202 mixed base positions at thirteen different DNA quantity ratios. The base positions analyzed were distributed across the region of the human mtDNA genome most often examined by forensic analysts. Analyses of peak height ratios revealed a highly significant correlation (R = 0.9998) between a change in the molar ratio of contributor DNA in a mixture and a change in peak height ratios in the resulting electropherogram. This strong correlation supports the use of comparative sequence analysis to determine the linkage phase (*i.e.*, the primary DNA base sequence) of the individual amplicons in a mixture.

Chapter 6: Separation of Mitochondrial DNA Mixtures by Denaturing High Performance Liquid Chromatography and Linkage Phase Analysis

§ 6 - 1 Introduction

Dye-terminator sequencing of DNA from hypervariable regions 1 and 2 (HV1/HV2) of human mtDNA is the current method of choice for the analysis of human mtDNA. A mixture of different mtDNA molecules in a single sample, however, presents a significant obstacle to successful mtDNA analyses by standard forensically validated methods. For example, an individual can naturally possess more than one mtDNA haplotype (*i.e.,* heteroplasmy) (Lightowlers *et al.*, 1997). Alternately, forensic analysts often encounter mixtures of DNA-containing samples from separate individuals (*i.e.*, a "situational" mixture). The ability to accurately determine the specific haplotypes of individual contributors to a DNA mixture can provide valuable investigative information.

While the size variation of short tandem repeats can facilitate the interpretation of nuclear DNA mixtures, this is not the case with mtDNA haplotypes differ primarily by base substitution or by only very small differences in length - most often arising from the insertion or deletion of a single base pair. Such subtle variations in sequence typically produce ambiguous base calls due to overlapping electrophoretic peaks. Similarly, variations in the length of mtDNA

molecules, such as those commonly associated with the cytosine stretches in both HV1 and HV2 result in long stretches of unreadable electropherograms. This reflects the fact that dye-terminated chains fall out of register with each other starting at the position of the insertion or deletion responsible for the length variant. In these cases, additional sequencing primers targeted to flank the heteroplasmic cytosine stretch must be used to obtain readable sequence. Unfortunately, this reduces the total amount of sequence data that can be used to characterize a given mitochondrial amplicon (Holland and Parsons, 1999). The ability to accurately resolve and interpret these types of samples in a timely and cost efficient manner would substantially increase the power of mtDNA analysis by allowing its use in cases where the current approach yields results that are of limited or no utility.

There are a number of molecular strategies that have been proposed to separate DNA mixtures. These include denaturing gradient gel electrophoresis (DGGE), single-strand conformational polymorphism (SSCP) analysis and subcloning into bacterial vectors (Hanekamp *et al.*, 1996; Barros *et al.*, 1997; Steighner *et al.*, 1999). Both DGGE and SSCP require manual recovery of fractionated DNA from polyacrylamide gels and PCR reamplification to generate enough template for DNA sequencing. Subcloning represents an even more time and labor-intensive approach. Furthermore, it would require forensic scientists to screen and sequence DNA from multiple transformed bacterial colonies to

ensure that observed sequence differences reflect genuine contributors to the starting template rather than artificial variants that were introduced as a result of DNA nucleotide misincorporation by *Taq* polymerase. Taken together, these shortcomings pose significant obstacles to the adoption of these technologies by forensic laboratories.

Pyrosequencing™ has recently been proposed as an alternate method for mixture analysis (Andreasson *et al.*, 2006). Pyrosequencing™ relies on the sequential addition of nucleotides to the reaction mixture resulting in light emission of the incorporated nucleotide (Ronaghi, 2001). In a mixed sample, a complicated pattern of peaks may allow for the determination of individual components of a mixture. Concerns regarding pyrosequencing™, however, include limited sequence reads (approx. 60-70bp) and, therefore, significant consumption of valuable sample in order to obtain complete coverage of the hypervariable region.

By comparison, denaturing high performance liquid chromatography (DHPLC) as described previously (Chapter 1) is a rapid and cost-effective method for physically fractionating mtDNA mixtures and then recovering amplicons for characterization by direct DNA sequencing (Emmerson *et al.*, 2003; Etokebe *et al.*, 2003). While doing so, DHPLC addresses the limitations of alternative strategies.

The underlying principle of DHPLC that is central to any effort to resolve an mtDNA mixture is Temperature Modulated Heteroduplex Analysis (TMHA) (Hou and Zhang, 2000). Briefly, when a mixture of two (or more) DNA amplicons is heat denatured and then allowed to slowly reanneal a combination of homo- and heteroduplices are formed. The homoduplices represent the original components of the mixture while the heteroduplices are generated by cross-hybridization of DNA strands from the different contributors to the mixture. Under partially denaturing conditions and an increasing gradient of a non-polar solvent (*e.g.*, acetonitrile), it is possible to physically separate the heteroduplices and the homoduplices from each other (Huber *et al.*, 1993). Heteroduplices, are inherently less stable as the result of one or more base pair mismatches and thus they appear as early eluting peaks in the chromatographic trace of an DHPLC assay (Huber *et al.*, 1995; O'Donovan *et al.*, 1998). The sensitivity of DHPLC to the thermodynamic stability of a DNA amplicon ensures that even very subtle sequence differences (*i.e.*, single nucleotide substitutions, insertions, deletions) between components of a mixture will be readily detected and fractionated in time (Kuklin *et al.*, 1997; Underhill *et al.*, 1997).

Analysis of PCR products by DHPLC integrates seamlessly into the established protocols for mtDNA sequencing used by the forensic community. Once fractionated, the recovered DNA requires no PCR reamplification, post PCR cleanup or quantitation by yield gel since PCR yield can be directly determined

from the height of the chromatographic elution peak (Chapter 3). Samples fractionated by DHPLC yield highly purified amplicons void of PCR amplification primers, enzymes or salts. Finally, sequencing results obtained from DHPLC purified samples result in highly reproducible electrophoretic peak patterns (Chapter 4), essential for accurate determination of DNA sequence identity.

Previous studies (Danielson *et al.*, 2007) have reported a correlation between the change in ratio of two contributors to a DNA mixture and the corresponding change in the ratio of electrophoretic peak heights at mixed-base positions. In the current study, the efficacy of DHPLC for the fractionation of mtDNA mixtures and automated recovery of sequence-ready DNA is investigated. Furthermore, a statistical analysis-based approach has been developed, which allows for the accurate determination of coordinated shifts in peak height ratios at mixed base positions (*i.e.*, linkage phase analysis). This serves as the basis for the determination of the haplotypes of the individual contributors to an mtDNA mixture.

§ 6 - 2 Materials and Methods

§ 6 - 2.1 Mitochondrial DNA Extraction and Quantification

This research was conducted in compliance with U.S. Federal Policy for the Protection of Human Subjects (56 FR 28003). Individual buccal swabs were collected from 46 unrelated subjects of diverse ethnicity who had previously

provided informed consent to participate in the study. Individual buccal swabs were incubated for 15min. in 10µL proteinase K (600mAU/ml) (Qiagen Inc., Valencia, CA) at 37°C, followed by DNA extraction using the EZ1 DNA Tissue kit on the BioRobot EZ1 DNA extraction robot (Qiagen Inc.) in accordance with the manufacturers' recommended protocol. Purified DNA was eluted into 200µL of nuclease free ddH_2O and following visual inspection and subsequent removal of residual paramagnetic beads, which have the potential to interfere with quantitative real-time PCR (qRT-PCR), samples were stored at -20°C.

All samples used in this study were quantified by qRT-PCR using previously published amplification primers and probe (Andreasson *et al.*, 2002) on a PRISM® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Quantification standards were prepared by serial dilution in $T_{10}E_{0.1}$/Glycogen buffer (10mM Tris-HCl pH 8.0; 0.1mM EDTA; 20µg/mL glycogen) of a previously subcloned 143bp mtDNA fragment encompassing the primer and probe binding sites. Seven serial dilutions ranging from $2.16x10^7$ – $2.1x10^1$ in ten-fold increments were used. Optimization of reaction conditions yielded a quantification assay in a total volume of 10µL containing 5µL TaqMan® Universal Master Mix – AmpErase® UNG (Applied Biosystems), 2µL DNA extract, 130nM probe and 100nM each primer. Samples were loaded onto MicroAmp® Optical 96-Well reaction plates and sealed using Optical Adhesive Covers (Applied

Biosystems). PCR amplification was initiated with 10 min incubation at 95°C followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min.

All standards were assayed in triplicate and all experimental samples were assayed in duplicate for statistical accuracy. Standard curves were generated and amplification plots were used to compare individual PCR reaction consistency, efficiency, and results. No-template DNA and reagent blank controls were also included in each assay.

§ 6 - 2.2 Mitochondrial DNA Amplification and Purification

Two-contributor mitochondrial DNA mixtures prepared at various quantity ratios ranging from 95:5-5:95 as determined by quantitative real-time PCR. Four forensically relevant regions of the human mitochondrial control region (*i.e.*, HV1A, HV1B, HV2A and HV2B) were amplified in 50µL reaction volumes. Each reaction was was prepared using 50pmol each of forensically-validated PCR primers (Wilson *et al.*, 1995; LaBerge *et al.*, 2003); 2.25U Ampli*Taq* GOLD® DNA polymerase (Applied Biosystems) supplemented with 0.25U *Pfu* DNA polymerase (Stratagene, La Jolla, CA); Ampli*Taq* GOLD® Buffer (Applied Biosystems); 10nmol of each dNTP (Stratagene) and 10µL of an approximately 10pg/µL human DNA extract. Amplifications were performed on a GeneAmp® 9700 thermocycler (Applied Biosystems) with an initial denaturation step at 95°C for 10 minutes, followed by 32 cycles of 95°C for 20 seconds, 60°C for 30 seconds

and 72°C for 45 seconds. The final extension at 72°C was for 15 minutes. PCR amplicons were purified and quantified at 50°C on a WAVE® 3500HT DNA Fragment Analysis System (Transgenomic Inc., Omaha, NE) containing a DNASep® analytical column (Transgenomic Inc.) packed with alkylated poly(styrenedivinylbenzene) resin (Huber *et al.*, 1993). DNA quantity was determined using peak area standard curve obtained from a dilution series of a six fragment quantitiation ladder (Chapter 3).

§ 6 - 2.3 DHPLC Mixture Fractionation

Cross-hybridization of mixed mtDNA amplicons was performed by denaturation at 95°C for 4 minutes and renaturation by gradual cooling (1.5°C/min) over a 45 minute period to reach a final temperature of 25°C (LaBerge *et al.*, 2003). Cross-hybridized mixtures were analyzed by Temperature-Modulated Heteroduplex Analysis (TMHA) (Kuklin *et al.*, 1997) at empirically predetermined optimal temperatures for each of the four forensic mtDNA amplicons (HV1A, 58°C; HV1B, 59.2°C; HV2A, 56.5°C and HV2B, 57°C.) The optimal acetonitrile linear gradients, generated from differential mixing of buffer A (0.1M TEAA) and buffer B (0.1M TEAA, 25% ACN), were 56% to 65% buffer B increase in 3.5 minutes for HV1A, HV2A and HV2B and a 55% to 64% buffer B increase in 3.5 minutes for HV1B. All samples were eluted at 0.9ml/min flow rate and detected by UV absorbance at 260nm. Eluted peaks were captured using an automated fraction collector (Transgenomic, Inc.); dried by vacuum

centrifugation for 30 minutes at 50°C followed by a 20-minute cool down to ambient on a CentriVap® Vacuum Concentrator (Labconco Corporation, Kansas City, MO). Samples were resuspended in nuclease-free H_2O; and stored frozen until DNA sequencing. Standard DHPLC controls included: zero-volume injections to screen for residual DNA carryover from the column matrix, no-template PCR controls to check for amplification contamination and manufacturer-supplied mutation control standards for buffer and column quality control.

§ 6 - 2.4 Mitochondrial DNA Sequencing

Sequencing reactions were prepared using the appropriate forensically-validated amplification primers. For greatest peak height consistency as previously determined (Chapter 4), 0.8ng total input DNA quantities were used for all labeling reactions and amplicons were extended according to the manufacturer's protocols using the BigDye® v1.1 Dye Terminator Cycle Sequencing kit (Applied Biosystems). Dye-terminated products were purified by Performa® DTR V3 96-well short plate purification columns (Edge BioSystems, Gaithersburg, MD) according to the manufacturer's protocol and resolved on a PRISM® 310 Genetic Analyzer (Applied Biosystems). Appropriate positive and negative controls were carried through the entire sample handling process to minimize cross contamination and to monitor amplification efficiency. The resulting sequence data were analyzed using the Sequencing Analysis Software v.5.1.1 (Applied Biosystems). The resulting .scf output file was then ported to the

CEQ™ 8000 Genetic Analysis System version 8.0 software package (Beckman-Coulter, Fullerton, CA) to allow output of the electrophoretic peak height data as a .txt file. Text files were then opened in Microsoft Excel for statistical analyses or displayed using the Sequencher™ v4.2 DNA analysis software (Gene Codes Corp, Ann Arbor, MI).

§ 6 - 3 Results

§ 6 - 3.1 Statistical Analyses of Mixture Ratios

A near perfect correlation (R = 0.9998) exists when mixtures are analyzed for changes in relative DNA quantities between two contributors versus changes in electrophoretic peak height ratios at mixed base positions (Chapter 5). This demonstrates that a change in peak height ratio of two components in a mixture is a reflection of an underlying change in relative DNA quantity at any mixed base position.

In these analyses, the relative shift in peak height fluorescence was calculated by comparing triplicate fluorescence intensity ratios across thirteen DNA quantity ratios (99:1 – 1:99) for each of 202 mixed nucleotide positions. Based on these data, exhaustive statistical analyses were performed using Tukey's honestly significant differences (HSD) test:

where, and are the mean of each group, is the mean square within for the group and is the number of samples per group. Using "R", "a language and environment for statistical computing and graphics" (http://www.r-project.org/), confidence levels were obtained for the 27,066 probability values.

The results of these analyses indicate an even tighter statistical correlation than presented above and have made it possible to precisely determine the level of statistical confidence associated with any detectible shift in electrophoretic peak height ratios. The calculated significance values for 27,066 comparisons of peak height fluorescence ratios illustrate the reliability of this relationship (Figure 6.1, Table 6.1). Based on these statistical calculations, as little as a 6% shift in fluorescence intensity of one contributor relative to the other makes it possible to accurately determine the linkage phase of the questioned mixture with a >99% confidence. Enrichment by 17% increases the statistical confidence of linkage phase to >99.9%.

§ 6 - 3.2 Linkage Phase Analysis of a Two-Component mtDNA Mixture

Because of the strong correlation between a change in the ratio of DNA quantities within a mixture and a change in the ratio of overlapping electrophoretic peaks, the linkage phase of individual amplicons present in a two-component mixture can be readily determined. This holds true even in cases

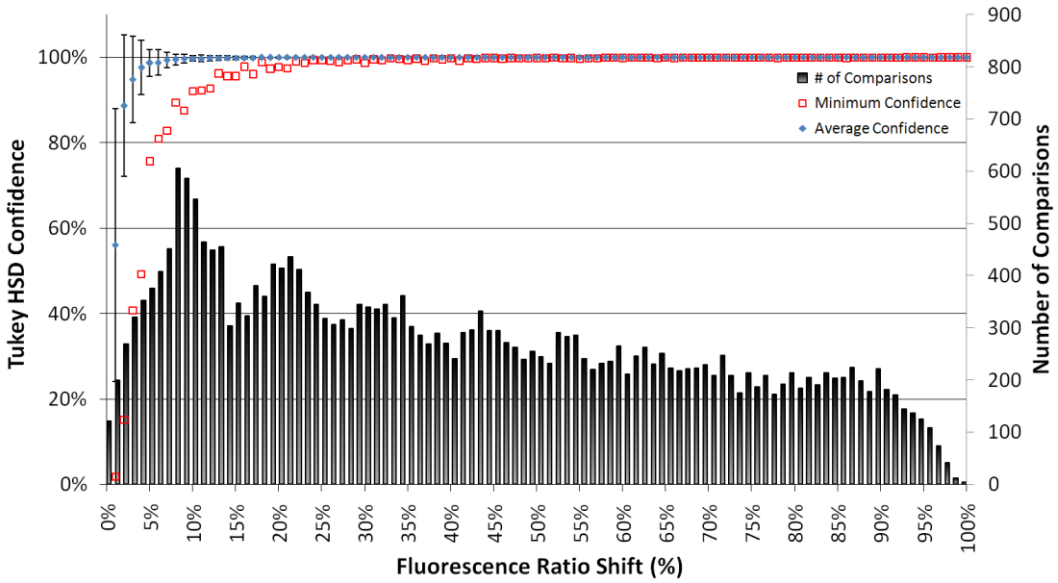
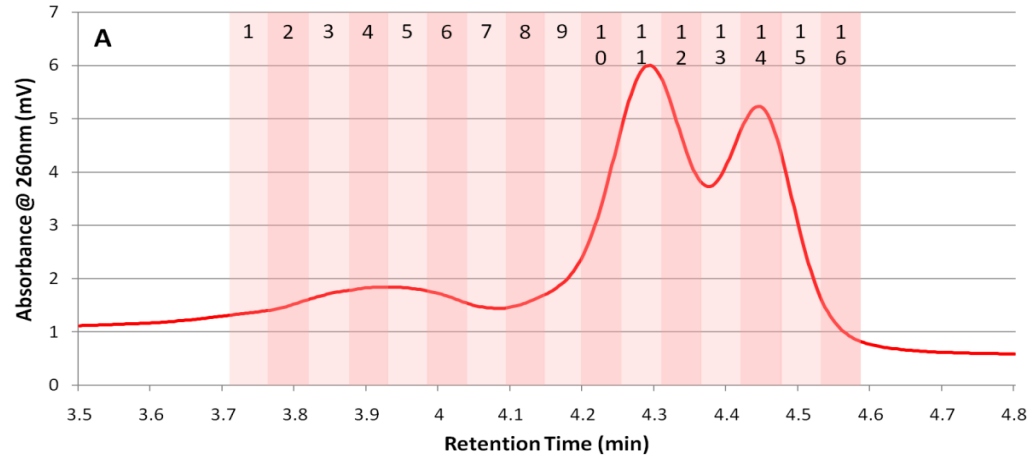


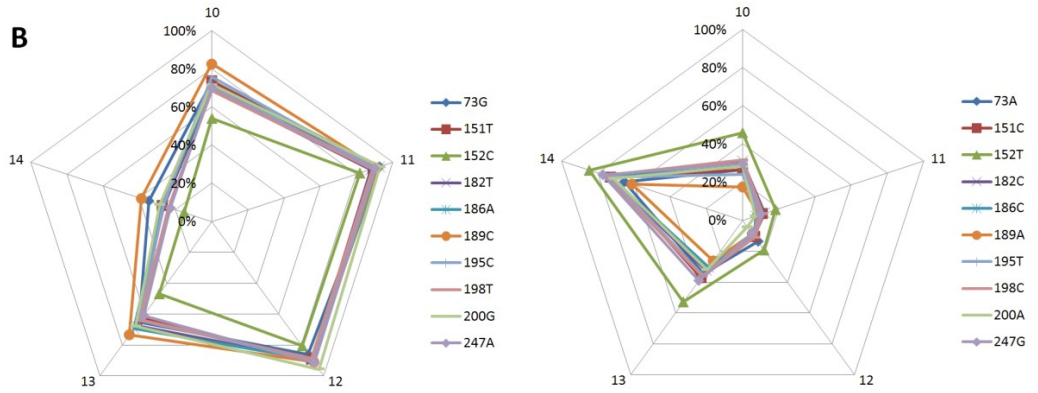
Figure 6.1: Tukey HSD confidence associated with changes in electrophoretic peak height ratios at mixed base positions. Shown are average and minimum confidence values (left y-axis) as a function of the percentage change in peak height fluorescence (x-axis) along with the number of data points behind each calculation (right y-axis). Greater than 99% confidence is obtained for a ≥6% shift in fluorescence while greater than 99.9% confidence is obtained for a ≥17% shift in fluorescence.

Table 6.1: Peak height enrichment and corresponding Tukey HSD confidence values. Shown for peak height enrichment level (measured as a percent) are the corresponding average and minimum Tukey HSD statistical confidence levels for linkage phase determination (*i.e.*, confidence that the shift represents an actual amplicon enrichment rather than stochastic peak height variation) as well as the number of comparisons performed for each enrichment percentage level.

	Confidence					Confidence			
Above Δ %	Average	Stdev	Minimum	# of Samples	Above Δ %	Average	Stdev	Minimum	# of Samples
0%	55.7068%	31.9128%	1.7756%	138	50%	99.9947%	0.0234%	99.7304%	243
1%	88.6051%	16.2347%	15.1027%	212	51%	99.9940%	0.0176%	99.8675%	240
2%	94.7428%	10.4752%	33.5045%	282	52%	99.9966%	0.0097%	99.9006%	292
3%	97.6763%	6.1475%	49.1783%	325	53%	99.9942%	0.0185%	99.8632%	285
4%	98.6880%	3.1613%	75.6875%	360	54%	99.9954%	0.0223%	99.6796%	284
5%	98.7216%	3.0254%	80.9047%	367	55%	99.9948%	0.0200%	99.7744%	239
6%	99.2914%	1.8050%	82.8608%	403	56%	99.9964%	0.0094%	99.9315%	228
7%	99.4059%	1.4048%	88.1837%	440	57%	99.9969%	0.0085%	99.9215%	232
8%	99.6674%	0.9882%	87.5446%	598	58%	99.9967%	0.0087%	99.9203%	234
9%	99.7234%	0.6783%	92.3707%	576	59%	99.9957%	0.0154%	99.8708%	253
10%	99.6906%	0.8978%	92.2587%	548	60%	99.9979%	0.0042%	99.9714%	216
11%	99.7749%	0.5440%	94.9600%	437	61%	99.9968%	0.0105%	99.9132%	252
12%	99.8023%	0.5937%	93.8351%	451	62%	99.9980%	0.0054%	99.9420%	257
13%	99.8681%	0.4183%	95.6135%	449	63%	99.9978%	0.0061%	99.9404%	231
14%	99.8634%	0.3680%	95.6200%	303	64%	99.9962%	0.0133%	99.8775%	252
15%	99.8760%	0.3074%	97.8045%	345	65%	99.9961%	0.0159%	99.7909%	227
16%	99.8866%	0.3532%	96.0773%	321	66%	99.9972%	0.0112%	99.8497%	206
17%	99.9419%	0.1445%	98.8909%	371	67%	99.9962%	0.0102%	99.9155%	223
18%	99.9522%	0.1116%	98.9438%	363	68%	99.9980%	0.0049%	99.9565%	230
19%	99.9531%	0.1613%	97.7573%	417	69%	99.9975%	0.0081%	99.9386%	228
20%	99.9528%	0.1581%	97.9136%	415	70%	99.9978%	0.0066%	99.9251%	200
21%	99.9647%	0.1233%	98.5116%	440	71%	99.9978%	0.0057%	99.9500%	250
22%	99.9680%	0.0919%	98.9741%	402	72%	99.9978%	0.0073%	99.9277%	209
23%	99.9680%	0.0771%	99.4915%	379	73%	99.9983%	0.0039%	99.9758%	174
24%	99.9720%	0.0823%	99.2918%	338	74%	99.9972%	0.0076%	99.9347%	212
25%	99.9713%	0.1067%	98.8324%	317	75%	99.9973%	0.0076%	99.9438%	182
26%	99.9734%	0.0927%	98.8315%	305	76%	99.9974%	0.0066%	99.9507%	209
27%	99.9713%	0.0752%	99.3188%	321	77%	99.9979%	0.0053%	99.9543%	173
28%	99.9788%	0.0863%	98.7722%	303	78%	99.9982%	0.0047%	99.9612%	184
29%	99.9770%	0.0897%	99.0558%	343	79%	99.9982%	0.0045%	99.9686%	216
30%	99.9814%	0.0475%	99.6072%	328	80%	99.9983%	0.0048%	99.9532%	173
31%	99.9858%	0.0388%	99.6518%	337	81%	99.9969%	0.0105%	99.8894%	195
32%	99.9894%	0.0300%	99.7626%	347	82%	99.9968%	0.0088%	99.9199%	199
33%	99.9875%	0.0372%	99.6480%	322	83%	99.9985%	0.0036%	99.9668%	204
34%	99.9858%	0.0578%	99.3888%	360	84%	99.9980%	0.0063%	99.9425%	202
35%	99.9908%	0.0220%	99.8495%	304	85%	99.9985%	0.0036%	99.9712%	211
36%	99.9868%	0.0535%	99.3068%	286	86%	99.9980%	0.0045%	99.9732%	220
37%	99.9878%	0.0409%	99.5278%	268	87%	99.9985%	0.0031%	99.9766%	197
38%	99.9860%	0.0513%	99.3155%	287	88%	99.9981%	0.0065%	99.9299%	186
39%	99.9902%	0.0372%	99.5235%	268	89%	99.9981%	0.0065%	99.9337%	237
40%	99.9926%	0.0156%	99.8707%	239	90%	99.9981%	0.0085%	99.8988%	188
41%	99.9913%	0.0289%	99.7386%	286	91%	99.9982%	0.0066%	99.9201%	170
42%	99.9927%	0.0241%	99.6614%	301	92%	99.9988%	0.0027%	99.9827%	157
43%	99.9938%	0.0163%	99.8699%	330	93%	99.9990%	0.0019%	99.9873%	140
44%	99.9940%	0.0172%	99.8543%	301	94%	99.9992%	0.0015%	99.9906%	131
45%	99.9927%	0.0267%	99.7099%	298	95%	99.9987%	0.0027%	99.9794%	115
46%	99.9927%	0.0206%	99.8491%	267	96%	99.9988%	0.0025%	99.9834%	75
47%	99.9942%	0.0184%	99.7539%	261	97%	99.9991%	0.0022%	99.9870%	42
48%	99.9948%	0.0169%	99.8216%	238	98%	99.9989%	0.0017%	99.9942%	15
49%	99.9953%	0.0131%	99.8587%	247	99%	99.9999%	0.0001%	99.9998%	4

where complete separation of individual components of a mixture cannot be achieved. In such cases, the linkage phase of each component of the mixture is determined by tracking the ratio of overlapping fluorescent peaks at all mixed base positions between two or more DHPLC fractions. The observation of coordinated shifts in the relative fluorescence ratios for a given set of nucleotides is consistent with them being in the same linkage phase and thus representing the same amplicon. Figure 6.2 represents the complete process of DHPLC fractionation followed by linkage phase analysis (LPA) of mixed nucleotide positions detected as a result of dye-terminator sequencing. A two-component mixture of HV2A amplicons containing ten mixed nucleotide positions (73G/A, 151T/C, 152T/C, 182T/C, 186A/C, 189C/A, 195C/T, 198T/C, 200G/A and 247A/G) was purified and fractionated into 16 individual fractions by DHPLC (Figure 6.2A). Five of the captured fractions (10-14) were sequenced and the mixed nucleotide positions were analyzed for relative peak height shifts (Figure 6.2B). Subsequently, these fractions were analyzed by LPA for determination of nucleotides corresponding to each contributor (Figure 6.2C). Separation of mixed nucleotides based on LPA identified the individual haplotypes of each of the two contributors to the mixture with greater than 99.99% average basecalling confidence. The haplotype of contributor 1 was 73A, 151C, 152T, 182C, 186C, 189A, 195T, 198C, 200A and 247G while that of contributor 2 was found to be 73G, 151T, 152C, 182T, 186A, 189C, 195C, 198T,





C

Linkage Phase Determination

Position	Comparison 11 and 14	Average Confidence	Confidence Std Dev	Minimum Confidence	Samples Behind Stats	Increasing	Decreasing
73	57.8%	99.997%	0.008%	99.921%	232	73A	73G
151	61.9%	99.997%	0.009%	99.913%	246	151C	151T
152	66.5%	99.998%	0.004%	99.966%	217	152T	152C
182	61.4%	99.997%	0.009%	99.913%	246	182C	182T
186	65.5%	99.997%	0.011%	99.866%	222	186C	186A
189	52.2%	99.996%	0.015%	99.817%	291	189A	189C
195	63.6%	99.996%	0.019%	99.749%	230	195T	195C
198	64.9%	99.996%	0.014%	99.878%	251	198C	198T
200	64.3%	99.996%	0.014%	99.878%	251	200A	200G
247	67.1%	99.997%	0.009%	99.918%	221	247G	247A

Figure 6.2: (**A**) Denaturing HPLC chromatogram, showing collected fractions, including those fractions used for dye-terminator sequencing (*i.e.*, fractions 10-14). (**B**) Radar graphs showing relative quantitation of fluorescence peak height ratios (y-axis) for 10 mixed base positions from sequence electropherograms representing DNA recovered from five DHPLC fractions (x-axis). (**C**) Linkage phase analysis determination and identification of individual contributors to a two-component mixture. Results are based on shifts in relative fluorescent intensity at all mixed base positions between DHPLC fractions 11 and 14.

200G and 247A. Although figures 6.2B and 6.2C illustrate only results obtained in the forward sequencing direction, confirmatory results were obtained when the same samples were sequenced in the reverse direction.

§ 6 - 3.3 Linkage Phase Analysis of Minor-Component mtDNA Mixtures

In forensic casework, mtDNA samples are not generally considered mixtures unless two or more nucleotide positions yield a secondary peak having a height of >10% that of the major component nucleotide. Based on results described in chapter 5, a 10% secondary peak height, however, can represent a molar mixture ratio of two contributors ranging anywhere from a 95:5 to 70:30 due to context specific incorporation of dye-terminators.

A known 95:5 mixture of HV2A, which appeared to be a single-source sample with a single heteroplasmic site, as determined by direct DNA sequencing, was separated into ten fractions by DHPLC and analyzed by LPA. Two of the isolated fractions yielded no mixture at all, while 5 yielded apparent heteroplasmy at one site as determined by the 10% threshold for secondary peak height. However, two of the fractions yielded 8 mixed-base positions where the height of the secondary peak ranged from 15% - 53% of that of the major peak. LPA analysis confirmed the existence of a mixture at all eight sites with an average confidence of 99.985%.

§ 6 - 3.4 Two-Component mtDNA Mixtures Analyzed by LPA

Individual mtDNA haplotypes for all samples used in this study were determined by dye-terminator sequencing prior to mixture generation. Thirteen samples, each of which represented a unique haplotype, were used to determine the accuracy of LPA for HV2A, (Table 6.2). Two-component mixtures were generated at multiple molar ratios ranging from 50:50 to 95:5 of contributor 1 to 2, respectively. All mixtures generated were PCR amplified and then fractionated by DHPLC. Selected fractions were then dye-terminator sequenced and the resulting electropherograms were then used for LPA. In every case, the results were in complete concordance with the known sequences from each contributor. Across all mixtures assayed, amplicon enrichment by DHPLC (as measured at individual mixed base positions) ranged from as little as a 2.1% to as much as a 92.3% shift in peak height fluorescence between individual DHPLC fractions (Table 6.3).

§ 6 - 3.5 Three- or More Component Mixtures

Mitochondrial DNA mixtures comprising three or more contributors are readily detected by DHPLC but yield significantly more complicated chromatographic traces than two-component mixtures. This reflects the significant increase in the number of heteroduplices that can be formed with each additional contributor. A total of 32 mixtures containing three to five distinct amplicons at various molar DNA input ratios were fractionated by

Table 6.2: The HV2A control region sequences of the samples used in the current study, shown as deviations from the revised Cambridge Reference Sequence (rCRS) (excluding 263A/G since all samples used in the study are 263G).

	64	72	73	146	150	151	152	182	185	186	188	189	195	198	200	217	222	225	226	228	239	242	247	249
rCRS*	**C**	**T**	**A**	**T**	**C**	**C**	**T**	**C**	**G**	**C**	**A**	**A**	**T**	**C**	**A**	**T**	**C**	**G**	**T**	**G**	**T**	**C**	**G**	**A**
1	T	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.
2	.	.	G	C	.	.	.	.	A	.	G	.	.	.	.	.	T	.	.	A	.	.	.	.
3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.
4	.	.	G	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	:
5	.	.	G	.	.	T	C	T	.	A	.	C	C	T	G	.	.	.	.	.	.	.	A	.
6	.	.	G	.	T	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
7	.	.	G	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
8	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
9	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
10	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	A	C	.	.	.	.	.
11	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
12	.	.	G	.	T	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.	.	.

*Except 263G

Table 6.3: Summary of HV2A mtDNA mixtures analyzed by DHPLC illustrating nucleotide enrichment and Tukey HSD-derived confidence ranges of mixed-nucleotide positions detected. Contributors identified in each mixture refer to the sequences characterized in table 6.2.

					All Sequenced Fractions		Maximum Δ Fluorescense				
Ratio	DHPLC peaks	Primer	Fractions Sequenced	Mixed sites detected	Ave Δ Fluorescence Range	Ave Δ Fluorescence	Δ Fluorescence Range	Confidence Ave.	Confidence Stdev.	Contributors	
50:50	4	C1	5	10	1.8% - 62.5%	30.1%	52.2% - 67.1%	99.997%	0.001%	rCRS	5
50:50	3	C1	6	10	2.1% - 59.1%	31.0%	33.5% - 75.0%	99.996%	0.003%	5	10
50:50	5	C1	4	3	1.0% - 62.2%	31.3%	60.9% - 63.0%	99.997%	0.001%	3	11
50:50	4	C1	7	4	3.6% - 57.1%	21.6%	54.0% - 65.0%	99.995%	0.0002%	rCRS	4
50:50	4	C1	6	5	1.7% - 74.6%	29.2%	71.3% - 77.9%	99.997%	0.0002%	1	7
50:50	2	C1	7	2	2.2% - 44.9%	19.7%	39.9% - 49.9%	99.994%	0.002%	8	1
50:50	4	C1	6	3	3.3% - 74.9%	33.7%	72.8% - 76.2%	99.998%	0.0002%	9	6
50:50	4	D2	6	10	1.3% - 74.4%	36.7%	57.8% - 92.3%	99.998%	0.001%	rCRS	5
50:50	2	D2	7	2	2.5% - 44.5%	19.7%	40.0% - 49.0%	99.993%	0.001%	8	1
50:50	4	D2	6	3	3.9% - 75.4%	36.0%	73.2% - 77.6%	99.998%	0.001%	9	6
60:40	4	C1	5	10	7.2% - 78.2%	44.2%	70.5% - 84.7%	99.998%	0.0004%	5	rCRS
60:40	4	C1	6	10	7.1% - 76.2%	39.3%	65.0% - 88.8%	99.997%	0.0004%	rCRS	5
60:40	4	C1	3	10	3.9% - 76.3%	51.0%	66.7% - 85.7%	99.998%	0.0003%	10	5
60:40	4	D2	6	10	3.8% - 72.9%	38.6%	60.4% - 79.7%	99.998%	0.0005%	5	rCRS
60:40	4	D2	6	10	7.1% - 71.1%	36.5%	56.4% - 79.3%	99.997%	0.001%	rCRS	5
60:40	4	D2	3	10	6.0% - 77.7%	52.7%	58.9% - 87.6%	99.998%	0.001%	10	5
70:30	4	C1	12	8	0.8% - 81.8%	34.2%	78.7% - 83.6%	99.998%	0.0004%	2	12
70:30	4	D2	12	8	1.1% - 82.0%	34.9%	73.7% - 87.1%	99.998%	0.0003%	2	12
80:20	4	C1	4	9	0.7% - 7.3%	4.5%	2.1% - 12.6%	98.922%	1.560%	5	rCRS
80:20	4	C1	5	10	0.9% - 46.0%	24.1%	34.7% - 54.1%	99.993%	0.002%	rCRS	5
80:20	4	C1	6	10	2.1% - 78.1%	32.2%	67.2% - 89.1%	99.998%	0.0005%	10	5
80:20	4	D2	5	10	0.6% - 29.5%	13.2%	17.8% - 45.2%	99.976%	0.016%	5	rCRS
80:20	4	D2	5	10	1.7% - 45.4%	24.0%	26.5% - 59.8%	99.992%	0.006%	rCRS	5
80:20	4	D2	5	10	1.9% - 22.8%	10.6%	5.7% - 37.5%	99.832%	0.379%	10	5
95:5	2	D2	10	8	0.4% - 34.5%	11.0%	26.7% - 43.8%	99.985%	0.007%	2	12
95:5	2	C1	12	8	0.3% - 40.8%	17.0%	26.8% - 47.8%	99.990%	0.007%	2	12

DHPLC. These multi-component mixtures yielded 4 to 10 clearly discernible chromatographic peaks.

Sequencing results and subsequent LPA of three component mixtures have shown that determination of one or two contributors can be achieved. However, application of statistical analyses for these samples remains difficult since shifting in peak height fluorescence ratio can be skewed as a result of multiple overlapping nucleotide contributions at any one mixed site. Reliable protocols for the resolution of three to five component mixtures by LPA, therefore, have not been developed.

§ 6 - 3.6 Identification of Heteroplasmic Components by LPA

Identification of heteroplasmy in mtDNA sequencing analysis has been proven to be of tremendous utility for human identification (Ivanov *et al.*, 1996). Due to the nature of DNA sequencing, low level heteroplasmic identification can often prove difficult to distinguish from underlying sequence chatter and noise. Utilizing DHPLC fractionation and LPA, identification of these can be made significantly easier, allowing for confirmation of suspected heteroplasmy in difficult samples. One sample displaying a broadened DHPLC profile revealed low level heteroplasmy at 16183. This sample was subjected to DHPLC fractionation and LPA, resulting in 25.6% and 24.0% enrichment of the minor component, depending on the direction of sequencing. This confirmed the existence of a

heteroplasmic 16183C/A with an average confidence of 99.973±0.091% and 99.968±0.077% depending on sequencing direction.

§ 6 - 4 Discussion

Based on aforementioned studies (Chapter 2) the accuracy of mixture screening by DHPLC is fully concordant with the detection of mixtures by direct sequencing. Furthermore, DHPLC may facilitate the detection of mixtures at ratios that might be missed by direct sequencing, *i.e.*, in cases where the quantity of the minor component is less than 10% that of the major component. The current study illustrates the potential utility of DHPLC in combination with LPA to resolve detected mixtures involving mtDNA amplicons commonly used by forensic analysts. DHPLC fractionation of a two-component mixture is independent of the initial screening of a sample to determine whether or not it consists of a mixture of non-identical amplicons.

DHPLC, allows an analyst to fractionate any DNA sample, regardless of whether it is a mixture or not. Subsequent sequencing analysis of the isolated fractions then allows for identification of the linkage phase of individual contributors and/or the detection of sequencing anomalies, *e.g.*, unincorporated dye blobs or suboptimal signal to noise ratio. Although fragment resolution by DHPLC, is a function of amplicon length and/or differences in primary sequence, it is difficult to predict *a priori* the characteristics of the chromatographic trace

that will result from DHPLC analysis of a specific mixture. Optimal separation and recovery is achieved when a two-component mixture can be resolved into four discernible peaks (*i.e.*, fully separated homo- and heteroduplices). This minimizes co-elution of incompletely resolved amplicons. When this is achieved, subsequent electropherograms are generally of good quality and are free of underlying electrophoretic signal from a second contributor. In practice, however, optimal chromatographic resolution is uncommon, and this typically results in the co-elution of target and non-target DNA amplicons. This produces a series of DHPLC fractions that are enriched for one contributor versus the other but which are not free of the second contributor. In the absence of complete separation, sequence electropherograms show signal from both contributors. As a consequence of this, overlapping electrophoretic peaks are produced at those nucleotide positions where the two amplicons differ in primary sequence.

Based on statistical analyses using Tukey's HSD test as little as a 6% shift in relative peak height fluorescence at mixed nucleotide positions is required to obtain a 99% statistical confidence in the linkage phase determination while a 17% shift in peak height ratio yields 99.9% confidence. Therefore, only a slight shift in peak height fluorescence is required in order to obtain a highly statistically significant linkage phase determination of a mixture. It is worth noting that the majority of mixtures analyzed to date show a >17% enrichment of one contributor over the other based on peak height shifts; thereby allowing for

>99.9% confidence in the resolution of the haplotypes of the individual contributors to two-component mtDNA mixtures. In the current study, all mixtures analyzed by LPA, including those showing as little as 2% enrichment, were accurately resolved albeit with reduced statistical confidence levels.

In the HV2A amplicon, two nucleotide positions within a single mixed sample were found after DHPLC fractionation to yield a <6% shift in peak height fluorescence. Even in this case however, LPA correctly determined the haplotypes of the two contributors. While, the average enrichment levels for the other three amplicons (HV1A, HV1B and HV2B) were lower than that of the HV2A amplicon, only 4 mixed-nucleotide positions, out of a total of 182 assayed, were resolved by LPA at a calculated confidence level of <99% (data not shown). In all cases, these represented mixtures where the ratio of the major to the minor contributor was 95:5, demonstrating that separation of these types of low-level mixtures cannot be obtained with high statistical confidence in every case. As a standard safeguard, therefore, enrichment of amplicons yielding a <6% (*i.e.*, <99% confidence) shift in peak height ratios should be treated with caution.

The tremendous value of DHPLC in combination with LPA is further demonstrated by the use of this approach to identify low level mixtures that might not be evident based on direct sequencing alone. By using DHPLC to selectively detect and enrich for minor contributors, it is possible to determine

not only that the original sample was a mixture, but also to determine the haplotype of the minor contributor to a high degree of statistical significance. One of the most important impacts of this in a forensic context is that samples thought to be of a single source or only slightly heteroplasmic as determined solely by direct DNA sequencing might actually contain low levels of a secondary contributor's DNA that can be enriched by DHPLC to provide potentially probative information. Until now, it has only been possible to identify the presence of additional contributors in these types of cases by the laborious process of subcloning mixed templates – a process that has not been forensically validated nor passed legal muster for courtroom admissibility. Based on the current studies, however, DHPLC represents the first reliable and forensically-validated method available to address low-level mixtures.

Studies to date on the use of DHPLC in combination with LPA for the deconvolution of three or more component mixtures make it possible in some cases to determine one and sometimes two major contributors. Because of skewed peak heights at mixed base positions, however, it has not been possible to reliably assign a concrete level of statistical confidence to the haplotype determinations in these cases. Further validation studies and development of more advanced LPA algorithms to handle multicomponenet mixtures are ongoing. DHPLC analysis of these types of mixtures, however, can yield significant information on the overall complexity of a mixture. From these

results, a forensic analyst can then determine whether to proceed with DHPLC fractionation and downstream sequencing. By giving analysts an early indication of the molecular complexity of a sample, it enables them to make informed decisions on whether or not to proceed with the more time, labor and cost intensive direct sequencing analyses and interpretation.

Although mtDNA analyses are generally only resorted to in cases where nuclear DNA analyses fail, DHPLC and LPA of mtDNA mixtures can be used to compliment short tandem repeat (STR) typing results obtained from mixtures. In cases where the minor contributor to a mixture only displays a partial profile, mtDNA mixture separation will yield confirmatory information regarding the two contributors to the mixture, regardless of their ratio. This can provide investigators with greater confidence in STR typing results from mixed populations.

Mixtures of mtDNA molecules have traditionally been viewed as irresolvable by the forensic community. The development of DHPLC fractionation of PCR products followed by LPA as a means of assigning a level of statistical confidence in the results obtained will have significant benefits for forensic practitioners. Courtroom admissibility of this approach, however, will rest upon exhaustive validation studies, including analyses of mixtures: consisting of DNA from a variety of tissue sources; recovered from a broad diversity of substrates

and; subjected to environmental insults likely to be encountered in a forensic context. Finally, the computationally tedious nature of LPA analyses have prompted the development of automated computer software for the identification and quantization of mixed nucleotide positions and the application of LPA statistics.

§ 6 - 5 Summary

A mixture of different mtDNA molecules in a single sample presents a significant obstacle to successful mtDNA analyses by standard forensically validated methods. The ability to accurately resolve and interpret these types of samples in a timely and cost efficient manner would substantially increase the power of mtDNA analysis by allowing its use in cases where the current approach yields results that are of limited or no utility. DHPLC is a rapid and cost-effective method for physically fractionating mtDNA mixtures; thereby allowing for amplicon recovery and subsequent characterization by direct DNA sequencing. This is achieved without the need for additional purification or re-amplification. The current study illustrates the efficacy of this approach. Further, a statistical analysis-based approach is presented (*i.e.*, linkage phase analysis), which allows for accurate determination of coordinated shift in peak height fluorescence at mixed base positions. This allows for identification of individual contributors to an mtDNA mixture with a high degree of statistical significance. The development

of linkage phase analysis in conjunction with DHPLC and direct DNA sequencing will have significant benefits for forensic practitioners working on mtDNA mixtures and will thereby increase the potential probative value of this class of physical evidence.

Chapter 7: Validation of Mitochondrial DNA Mixture Separation by Linkage Phase Analysis

§ 7 - 1 Introduction

The use of mitochondrial DNA sequencing has been proven to be of tremendous utility in forensic investigations and has been admitted to court on several occasions (See www.denverda.org). Until recently, however, mixtures of two or more contributors would have complicated mitochondrial DNA haplotype analysis and would have often yielded uninformative sequencing data. This has limited the statistical power of discrimination of mtDNA analyses. Fractionation of mtDNA mixtures by Denaturing High Performance Liquid Chromatography (DHPLC) and subsequent Linkage Phase Analysis (LPA) has demonstrated the reliability and effectiveness by which mixed samples can be resolved.

The use of DHPLC has been shown to facilitate the fractionation and recovery of PCR amplicons and in some cases has yielded complete separation of the individual components of mixtures of two mtDNA contributors. This is achieved without reamplification or excessive processing of PCR products as to minimize cross contamination or amplification of contaminating components. In cases where only partial separation of the individual contributors to a mixture is achieved, LPA can be used to deconvolve mixed nucleotide positions. This allows

for the identification of individual haplotypes in the mixture (Chapter 6). The method also allows for a statistical weight to be assigned to base calls associated with the separated mixtures.

The aim of the current study, therefore, was to validate the use of DHPLC fractionation and LPA of sequencing results obtained from these fractions as a combined method for mtDNA mixture analysis. This study was performed using a variety of commonly encountered forensic samples as well as environmentally insulted samples to facilitate the use of this technology in a forensic context. This study was in accordance with the DNA Advisory Board (DAB) developmental validation standards for sensitivity, reproducibility, accuracy, *etc*. Furthermore, recognizing that Daubert v. Merrell Dow Pharmaceuticals, Inc., 509 U.S. 579, 593-94 (1993), Frye's "general acceptance" test, Frye v. United States, 293 F. 1013, 1014 (D.C. Cir. 1923) and federal rules of evidence specifically Rules CRE 403 and CRE 702 provide the standard for admitting scientific evidence in the federal courts. The experimental design of the validation assays was planned with both the Frye and Daubert standards in mind.

The current study shows that mtDNA mixture fractionation by DHPLC and sequence analysis by LPA is a valid, highly reliable and rapid method for mtDNA mixture separation and subsequent haplotype determination of individual contributors to a mixed sample.

§ 7 - 2 Materials and Methods

§ 7 - 2.1 Sample Preparation and DNA Extraction

This research was conducted in compliance with U.S. Federal Policy for the Protection of Human Subjects (56 FR 28003). Individual samples, including blood, saliva, semen and hair were collected from unrelated subjects who had previously provided informed consent to participate in the study. The mtDNA haplotypes of all sample donors were determined separately by sequencing of individual buccal swabs. No two donors had identical mtDNA sequences for the hypervariable regions used in the study.

Combinations of undiluted body fluid mixtures (*i.e.*, blood, saliva and semen) (5µL each) were used to stain a variety of substrates including denim, leather, wood, carpet, nylon and wallboard. All samples were aged for four weeks at room temperature, prior to DNA extraction. In order to assess the potential impact of environmental contaminants, combinations of undiluted body fluid mixtures, (*i.e.*, blood, saliva and semen) (5µL each) were applied to Pur-Wraps® sterile cotton tipped applicators (Hardwood Products, Guilford, ME) that had previously been dipped in gasoline, a slurry of sterile water and soil, used motor oil, laundry detergent, 1N sodium hydroxide and 10% glacial acetic acid and allowed to dry for 2 hours.

Hair samples (natural, dyed dark brown or permed, axial hair, pubic hair; 2cm; without root) were mixed with 10µL whole blood, semen or saliva prior to

two 56°C, six hour incubations. Aged human bones (generously provided by Dr. Randall Skelton, University of Montana, Missoula, MT) were cleaned twice in 25ml of 5% Tergazyme™ and sonicated for 30-45 min.. After rinsing in 95% EtOH the bones were allowed to dry overnight at room temperature. All bone samples were then pulverized using the SPEX CertiPrep® 6850 Freezer/Mill in accordance with manufacturers' recommended protocol for small bone samples. Briefly, samples were placed in SPEX CertiPrep® 6751 grinding vials and frozen in liquid nitrogen for 10min. Samples were then pulverized in three 4min. cycles (2min grinding period, 2min cooling period). Following retrieval from the grinding vial, 200mg of bone powder was mixed with 700µL EDTA (0.5M, pH 8.5) and incubated at 37°C for 24 - 48 hours. Equal amounts of bone powder (200g) obtained from additional samples or 20µL of whole blood were mixed together along with 20µL proteinase K (600mAU/mL) (Qiagen, Inc.) and incubated at 56°C for at least 3 hours.

All DNA samples were extracted on the BioRobot EZ1 using the EZ1 DNA tissue kit (Qiagen Inc., Valencia, CA) in accordance with the manufacturers' recommended protocol for DNA extraction from trace samples. In order to maximize DNA recovery, all samples used in this study were incubated for 15min. at 56°C in 190µL of G2 Buffer and 10µL proteinase K (600mAU/mL) (Qiagen, Inc.) prior to extraction, unless otherwise noted. Extracted DNA was eluted into 200µL of nuclease free ddH_2O and stored at -20°C until PCR amplification.

§ 7 - 2.2 Mitochondrial DNA Amplification and Purification

Mitochondrial DNA hypervariable region amplification reactions (50µL) were prepared using 50pmol each of forensically-validated PCR primer pairs for relevant regions of the human mitochondrial control region (*i.e.,* HV1A, HV1B, HV2A and HV2B amplified by primer sets, A1/B2, A2/B1, C1/D2 and C2/D1, respectively) (Wilson *et al.,* 1995; Budowle *et al.,* 2000). For each reaction, 2.25U Ampli*Taq* GOLD® DNA polymerase (Applied Biosystems, Foster City, CA) supplemented with 0.25U *Pfu* DNA polymerase (Stratagene, La Jolla, CA); Ampli*Taq* GOLD® Buffer (Applied Biosystems); 10nmol of each dNTP (Stratagene) and 10µL of an approximately 10pg/µL DNA extract were used. Amplifications were performed on a GeneAmp® 9700 thermocycler (Applied Biosystems) with an initial denaturation at 95°C for 10 minutes, followed by 32 cycles of 95°C for 20 seconds, 60°C for 30 seconds and 72˚C for 45 seconds. The final extension at 72°C was for 15 minutes. PCR amplicons were purified and quantified at 50°C on a WAVE® 3500HT DNA Fragment Analysis System (Transgenomic Inc., Omaha, NE) containing a DNAsep® analytical column packed with alkylated poly(styrenedivinylbenzene) resin (Huber *et al.,* 1993) using previously established methods (Chapter 3).

§ 7 - 2.3 DHPLC Mixture Fractionation

Cross hybridization of mixed amplicons was performed by denaturation at 95°C for 4 minutes and renaturation by gradual cooling (1.5°C/min) over a 45

minute period to reach a final temperature of 25°C (LaBerge *et al.,* 2003). Cross hybridized mixtures were then analyzed by Temperature-Modulated Heteroduplex Analysis (TMHA) (Kuklin *et al.,* 1997) using a DNASep® column in the presence of 0.1M triethylammonium acetate (TEAA) pH 7.0 as an ion pairing reagent. Empirically predetermined optimal temperatures for each of the four forensic mtDNA amplicons (HV1A, 58°C; HV1B, 59.2°C; HV2A, 56.5°C and HV2B, 57°C) were used for each assay. The optimal acetonitrile linear gradients generated from differential mixing of buffer A (0.1M TEAA) and buffer B (0.1M TEAA, 25% ACN) were 56% to 65% buffer B increase in 3.5 minutes for HV1A, HV2A and HV2B and a 55% to 64% buffer B increase in 3.5 minutes for HV1B. All samples were eluted at a 0.9ml/min flow rate and detected by UV absorbance at 260nm. Eluted peaks were captured using an automated fraction collector (Transgenomic, Inc.); dried by vacuum centrifugation for 30 minutes at 50°C followed by a 20-minute cool down to ambient temperature on a CentriVap® Vacuum Concentrator (Labconco Corporation, Kansas City, MO). Samples were resuspended in nuclease-free H_2O; and stored frozen until DNA sequencing. Standard DHPLC controls included: zero-volume injections to screen for residual DNA carryover from the column matrix, no-template PCR controls to check for amplification contamination and manufacturer provided mutation control standards for buffer and column quality control.

§ 7 - 2.4 Mitochondrial DNA Sequencing

DNA sequencing reactions were prepared using the appropriate forensically-validated amplification primers. For greatest peak height consistency as previously determined (Chapter 4), 0.8ng total input DNA quantities were used for all labeling reactions and amplicons were extended according to the manufacturer's protocols using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Dye-terminated products were purified by Performa® DTR V3 96-well Short Plate Kit (Edge BioSystems, Gaithersburg, MD) according to the manufacturer's protocol and resolved on a PRISM® 310 Genetic Analyzer (Applied Biosystems) using POP-6™ polymer and 47cm x 50µm capillaries (Applied Biosystems). Appropriate positive and negative controls were carried through the entire sample handling process to minimize cross contamination and to monitor amplification efficiency. Raw electrophoretic traces were analyzed using the KB Basecaller (Applied Biosystems) together with the appropriate dye set mobility file for the v1.1 kit using Sequencing Analysis Software v.5.1.1 (Applied Biosystems). The resulting sequence data were analyzed using the Sequencher™ v4.2 DNA analysis software (Gene Codes Corp, Ann Arbor, MI) and individual peak height values obtained using the CEQ™ 8000 Genetic Analysis System version 8.0 software package (Beckman-Coulter, Fullerton, CA)

§ 7 - 2.5 Linkage Phase Analysis

Mixed nucleotide positions were considered to be any positions characterized by a primary peak and an underlying secondary peak having a height >10% that of the major peak as determined by inspection of the relative fluorescent units (RFU) in the analyzed data. Corresponding mixed nucleotide positions were analyzed between fractions and LPA was used to resolve mixtures based on a comparison of those fractions showing the greatest average change in fluorescence ratio across all mixed base positions. For each mixed nucleotide position, the change in the base fluorescence ratio corresponds to a change in amplicon quantity between the two components that make up the mixture. The statistical confidence of this relationship was derived from statistical analyses of data gathered during the developmental validation study. These data were obtained from 347 comparisons of 13 different contributor ratios at 202 unique nucleotide positions across the hypervariable region of the mtDNA genome, for a total of 27,066 data points (Chapter 6).

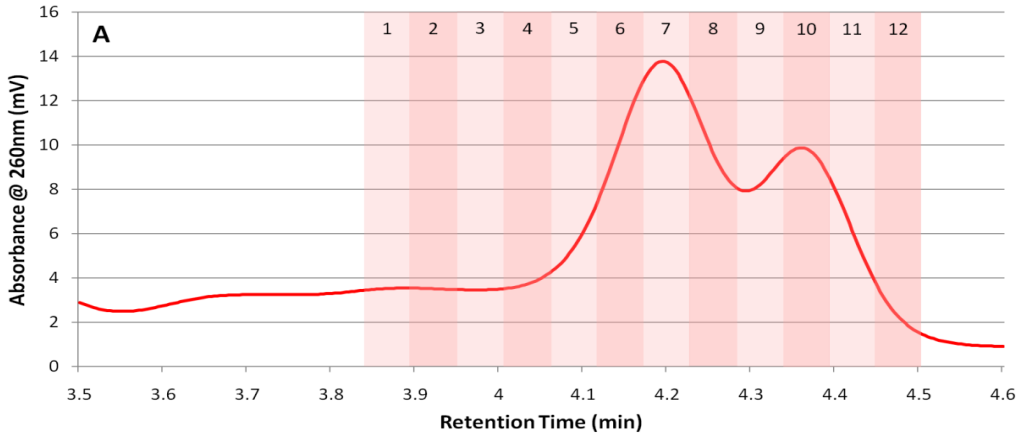
§ 7 - 3 Results and Discussion

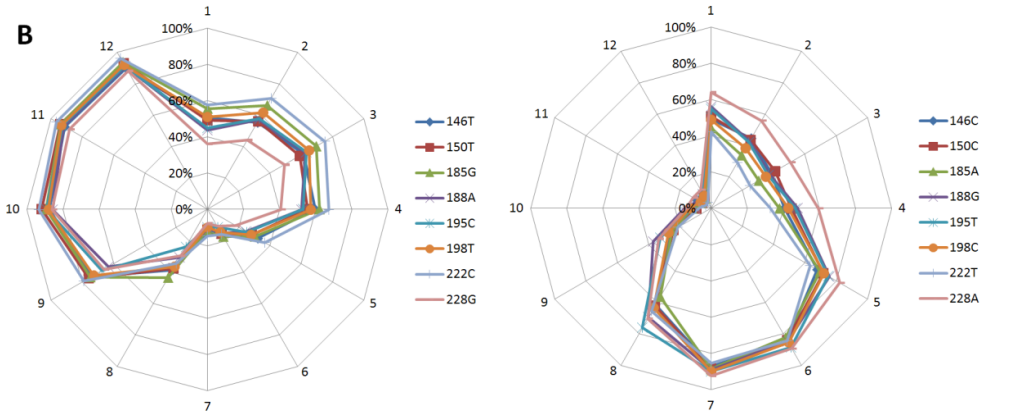
§ 7 - 3.1 Efficiency of Component Identification as a Function of Chromatographic Trace Characteristics

As a reflection of chromatographic separation of non-identical amplicons, DHPLC trace characteristics should be correlated with the efficiency of mixture resolution. Mixture analysis by TMHA, yields chromatographic traces ranging

from a major peak complimented by a significant shoulder, to multiple peaks representing each of the homo- and heteroduplices formed during denaturation and subsequent renaturation of the DNA present in the sample. In a two-component mixture, the optimal DHPLC trace displays complete separation of each homo- and heteroduplex peaks.

Two-component mixtures which display optimal four peak resolution, where the two homoduplices resolve into two distinct peaks of close or equal height, generally yield fractions that, when sequenced, display the most pronounced coordinated shift in peak height fluorescence ratios. Figure 7.1 illustrates the fractionation and LPA analysis of a two-component mixture with four peak resolution. The two homoduplices were captured by automated fraction collection (Figure 7.1A) and peak height ratios determined by dye terminator sequencing (Figure 7.1B). Pair-wise comparison of fluorescence ratios at mixed base positions across all fractions (66 comparisons among the 12 fractions recovered), yielded an average peak height ratio shift of 34.2 ± 21.1%. Of these, the comparison of fractions 7 and 11 yields the greatest coordinated shift in peak height fluorescence ratios across all mixed bases, with an average of 81.8 ± 1.5%. This was associated with linkage phase determination where the base calling confidence was greater than 99.99% for all mixed base positions (Figure 7.1C). Comparison of fractions 11 and 12 yields the least shift in peak height fluorescence ratios, with a mixed base average of 0.77 ± 0.47%. The





C

Linkage Phase Determination

Position	Comparison 7 and 11	Average Confidence	Confidence Std Dev	Minimum Confidence	Samples Behind Stats	Increasing	Decreasing
146	78.7%	99.998%	0.007%	99.945%	192	146T	146C
150	82.8%	99.998%	0.006%	99.950%	190	150T	150C
185	82.4%	99.998%	0.006%	99.950%	190	185G	185A
188	81.3%	99.998%	0.006%	99.962%	204	188A	188G
195	82.7%	99.998%	0.006%	99.950%	190	195C	195T
198	83.6%	99.998%	0.004%	99.967%	214	198T	198C
222	82.2%	99.998%	0.006%	99.950%	190	222C	222T
228	80.8%	99.998%	0.003%	99.979%	184	228G	228A

Figure 7.1: (**A**) Denaturing HPLC chromatogram, showing the twelve collected fractions all of which were used for dye-terminator DNA sequencing. (**B**) Relative quantitation of fluorescence peak height ratios (y-axis) for eight mixed base positions from sequence electropherograms representing DNA recovered from all DHPLC fractions collected (x-axis). (**C**) Use of linkage phase analysis to determine the haplotypes of the individual contributors to this two-component mixture. This analysis is based on shifts in peak height ratios at mixed base positions between DHPLC fractions 7 and 11.

particularly low shift in peak height ratio between fractions 11 and 12 is due to the fact that both of these fractions represent the near complete isolation of a single contributor to the mixture – the same contributor in both cases. A comparison between these two fractions, therefore, adds no value to the overall analysis of the mixture. Furthermore, the shift in peak height between fractions 11 and 12 is comparable to the internal variability noted in replicate sequencing reactions of mixtures consisting of the same amplicon ratios (Chapter 5) as well as the inherent peak height variability of individual peaks in any sequencing reaction (Chapter 4).

Although fractions 7 and 11 yielded the greatest amount of fluorescence ratio shift across all mixed nucleotides, LPA of the majority of fraction comparisons (49 out of 66) yielded linkage phase determinations with an average base calling confidence greater than 99.9%. The remaining 17 fraction comparisons, where average base calling confidence was less than 99.9%, reflected two phenomena. The first of these is the comparison of adjoining fractions containing very similar molar ratios of mixed amplicons (*e.g.*, fractions 6 and 7), or fractions flanking the same peak which again would be expected to possess similar amplicons ratios (*e.g.*, fractions 5 and 8).

Table 7.1 illustrates the results obtained from 51 two-component mixtures at component molar ratios ranging from 50:50 to 95:5. These samples

Table 7.1: Summary of mtDNA mixtures ratios analyzed by DHPLC including change in peak height ratios attained and base calling confidence ranges for linage phase determination at mixed nucleotide positions.

						All Sequenced Fractions		Maximum Δ Fluorescense		
Sample	Ratio	DHPLC peaks	Primer	Fractions Sequenced	Mixed sites detected	Ave Δ Fluorescence Range	Ave Δ Fluorescence	Δ Fluorescence Range	Confidence Ave.	Confidence Stdev.
CS-1	50:50	3	A1	4	4	6.2% - 55.2%	29.7%	52.7% - 57.7%	99.996%	0.001%
CS-2	50:50	3	A1	4	2	1.1% - 10.4%	5.4%	9.3% - 11.6%	99.754%	0.033%
CS-3	50:50	4	A2	7	3	0.7% - 44.4%	19.5%	42.5% - 47.2%	99.993%	0.001%
CS-4	50:50	3	B1	7	8	2.1% - 26.0%	13.1%	19.8% - 30.2%	99.974%	0.009%
CS-5	50:50	4	B1	7	5	3.0% - 31.6%	15.2%	21.6% - 39.2%	99.982%	0.010%
CS-6	50:50	3	B2	3	4	4.2% - 8.3%	5.7%	4.8% - 10.0%	99.451%	0.526%
CS-7	50:50	3	B2	5	2	1.8% - 18.8%	8.8%	13.5% - 24.0%	99.923%	0.070%
CS-8	50:50	4	C1	5	10	1.8% - 62.5%	30.1%	52.2% - 67.1%	99.997%	0.001%
CS-9	50:50	3	C1	6	10	2.1% - 59.1%	31.0%	33.5% - 75.0%	99.996%	0.003%
CS-10	50:50	5	C1	4	3	1.0% - 62.2%	31.3%	60.9% - 63.0%	99.997%	0.001%
CS-11	50:50	4	C1	7	4	3.6% - 57.1%	21.6%	54.0% - 65.0%	99.995%	0.0002%
CS-12	50:50	4	C1	6	5	1.7% - 74.6%	29.2%	71.3% - 77.9%	99.997%	0.0002%
CS-13	50:50	2	C1	7	2	2.2% - 44.9%	19.7%	39.9% - 49.9%	99.994%	0.002%
CS-14	50:50	4	C1	6	3	3.3% - 74.9%	33.7%	72.8% - 76.2%	99.998%	0.0002%
CS-15	50:50	4	D2	6	10	1.3% - 74.4%	36.7%	57.8% - 92.3%	99.998%	0.001%
CS-16	50:50	2	D2	7	2	2.5% - 44.5%	19.7%	40.0% - 49.0%	99.993%	0.001%
CS-17	50:50	4	D2	6	3	3.9% - 75.4%	36.0%	73.2% - 77.6%	99.998%	0.001%
CS-18	60:40	2	B1	6	7	3.1% - 28.5%	15.3%	21.0% - 35.9%	99.977%	0.012%
CS-19	60:40	2	B1	3	8	7.8% - 22.5%	15.0%	16.3% - 27.0%	99.959%	0.027%
CS-20	60:40	4	C1	5	10	7.2% - 78.2%	44.2%	70.5% - 84.7%	99.998%	0.0004%
CS-21	60:40	4	C1	6	10	7.1% - 76.2%	39.3%	65.0% - 88.8%	99.997%	0.0004%
CS-22	60:40	4	C1	3	10	3.9% - 76.3%	51.0%	66.7% - 85.7%	99.998%	0.0003%
CS-23	60:40	4	C2	3	5	4.0% - 24.4%	16.5%	16.7% - 32.5%	99.960%	0.038%
CS-24	60:40	4	C2	10	6	1.8% - 70.3%	25.3%	58.5% - 85.1%	99.997%	0.001%
CS-25	60:40	4	D2	6	10	3.8% - 72.9%	38.6%	60.4% - 79.7%	99.998%	0.0005%
CS-26	60:40	4	D2	6	10	7.1% - 71.1%	36.5%	56.4% - 79.3%	99.997%	0.001%
CS-27	60:40	4	D2	3	10	6.0% - 77.7%	52.7%	58.9% - 87.6%	99.998%	0.001%
CS-28	70:30	2	A1	13	4	0.3% - 29.9%	13.0%	24.5% - 35.8%	99.980%	0.008%
CS-29	70:30	2	A1	13	4	0.4% - 30.2%	13.8%	27.3% - 34.3%	99.979%	0.006%
CS-30	70:30	3	A1	7	2	1.4% - 26.3%	11.5%	20.1% - 32.4%	99.975%	0.022%
CS-31	70:30	2	B2	7	2	0.9% - 26.2%	12.9%	26.0% - 26.3%	99.974%	0.001%
CS-32	70:30	2	B2	9	2	0.4% - 32.0%	13.0%	26.9% - 37.1%	99.982%	0.011%
CS-33	70:30	3	B2	10	2	0.7% - 25.1%	11.1%	24.5% - 25.7%	99.973%	0.0004%
CS-34	70:30	4	C1	12	8	0.8% - 81.8%	34.2%	78.7% - 83.6%	99.998%	0.0004%
CS-35	70:30	3	C2	7	4	1.1% - 15.3%	7.8%	12.1% - 17.6%	99.897%	0.054%
CS-36	70:30	4	D2	12	8	1.1% - 82.0%	34.9%	73.7% - 87.1%	99.998%	0.0003%
CS-37	80:20	2	B1	7	7	1.9% - 20.8%	10.3%	15.2% - 27.9%	99.943%	0.039%
CS-38	80:20	4	C1	4	9	0.7% - 7.3%	4.5%	2.1% - 12.6%	98.922%	1.560%
CS-39	80:20	4	C1	5	10	0.9% - 46.0%	24.1%	34.7% - 54.1%	99.993%	0.002%
CS-40	80:20	4	C1	6	10	2.1% - 78.1%	32.2%	67.2% - 89.1%	99.998%	0.0005%
CS-41	80:20	3	C2	6	6	2.6% - 38.5%	21.8%	22.3% - 55.6%	99.984%	0.014%
CS-42	80:20	4	D2	5	10	0.6% - 29.5%	13.2%	17.8% - 45.2%	99.976%	0.016%
CS-43	80:20	4	D2	5	10	1.7% - 45.4%	24.0%	26.5% - 59.8%	99.992%	0.006%
CS-44	80:20	4	D2	5	10	1.9% - 22.8%	10.6%	5.7% - 37.5%	99.832%	0.379%
CS-45	90:10	3	A2	7	3	0.7% - 18.3%	7.6%	8.0% - 26.1%	99.871%	0.167%
CS-46	95:5	2	A1	7	2	0.5% - 26.8%	12.3%	19.0% - 34.6%	99.967%	0.030%
CS-47	95:5	2	A2	7	3	0.4% - 12.9%	5.1%	5.4% - 18.5%	99.525%	0.663%
CS-48	95:5	2	B2	7	2	0.3% - 22.1%	8.6%	11.2% - 33.0%	99.884%	0.151%
CS-49	95:5	2	C2	7	4	0.2% - 4.5%	1.6%	3.4% - 6.5%	98.557%	0.710%
CS-50	95:5	2	D2	10	8	0.4% - 34.5%	11.0%	26.7% - 43.8%	99.985%	0.007%
CS-51	95:5	2	C1	12	8	0.3% - 40.8%	17.0%	26.8% - 47.8%	99.990%	0.007%

display a range of DHPLC trace characteristics, from one major peak and a corresponding shoulder, to complete separation of all four homo- and heteroduplex peaks. All amplified samples were fractionated accordingly by DHPLC under partially denaturing conditions. Three to twelve fractions for each sample were then labeled for direct sequencing. All sequenced fractions were analyzed for mixed base positions. These were compared across fractions by LPA in order to identify those bases that displayed coordinated shifts in peak height fluorescence ratios. Eighty-two percent of analyzed mixtures yielded linkage phase determinations with average base calling confidence of greater than 99.9%, when the fractions that were compared were those that displayed the greatest shift in peak height fluorescence. Of the nine remaining mixtures, only two yielded linkage phase determinations with an average base calling confidence of less than 99% (CS-38; 98.922% and CS-49; 98.557%).

Analysis of sample CS-38 by DHPLC using TMHA (Figure 7.2) was characterized by separation of the homoduplices into a major peak and a trailing shoulder (separated into six 50µL fractions). The heteroduplices (not captured) formed to only a minimal degree as evidence by their low elution peak heights. The major reason for this lack of apparent separation for CS-38 when analyzed by LPA is that the trailing edge of the shoulder was not sequenced (fractions 5 and 6). When compared to sequencing results obtained from the opposite sequencing primer (CS-42; where fractions 1-5 were sequenced), the shift in

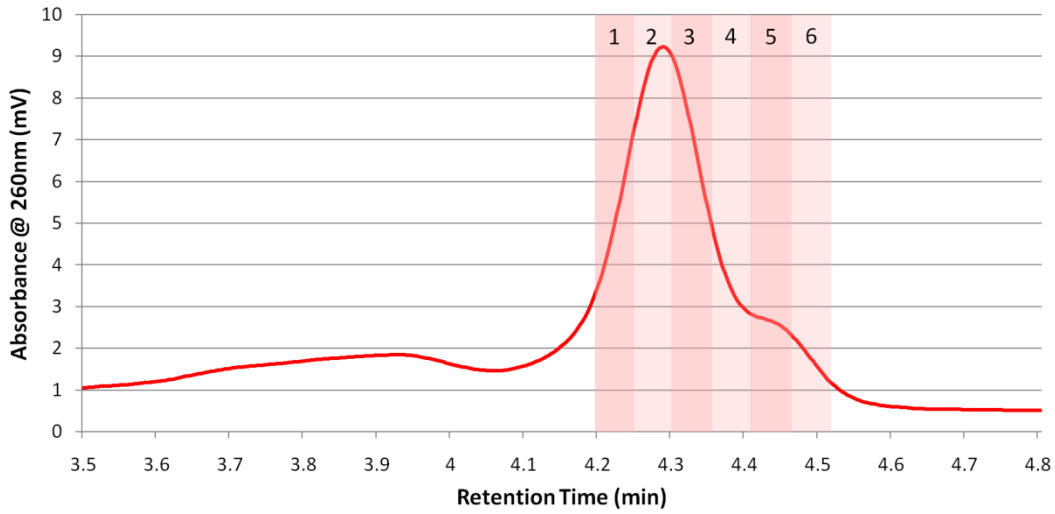


Figure 7.2: Analysis of sample CS-38 by TMHA, showing separation of individual homoduplices into a major peak (fractions 1-3) and a trailing shoulder (fractions 5 and 6). Also shown is the formation of minor heteroduplices (not captured) at a retention time of approximately 3.9 minutes. Linkage phase analysis of fractions 1 and 4 yielded separation of the two contributors to the mixture with an average base calling confidence of 98.922%. Analysis of fractions 1 and 5 yielded an average confidence of 99.976%.

peak height fluorescence was significantly greater. These results illustrate that, while blind fraction sequencing yields significant information as to the haplotypes comprising the mixture, considerably improved results are attained when strategic fraction sequencing is performed.

Analysis of sample CS-49 by TMHA (Figure 7.3) shows the separation and capture of a single broad homoduplex peak (fractions 4-7) preceded by a single heteroduplex peak (fractions 1-2). This was primarily due to the nature of the mixture itself (a 95:5 component ratio) in which the major contributor peak masks the minor contributor peak. As a result, comparisons of mixed base positions in the sequencing data obtained from these fractions revealed only small shifts in peak height ratios. Of these, the largest coordinated shift in peak height fluorescence ratios was seen between fractions 4 and 7. LPA of these fractions, allowed for the determination of the linkage phase of each of the two contributors to this mixture. This illustrates, that the broad nature of the homoduplex peak is due to a slight difference in the retention times between the two homoduplices present in the mixture. The LPA results clearly indicate that even when retention time differences are not readily apparent (as the minor component homoduplex is hidden by the major component peak), the subtle variation in retention time can be detected by LPA of the sequences obtained from these fractions.

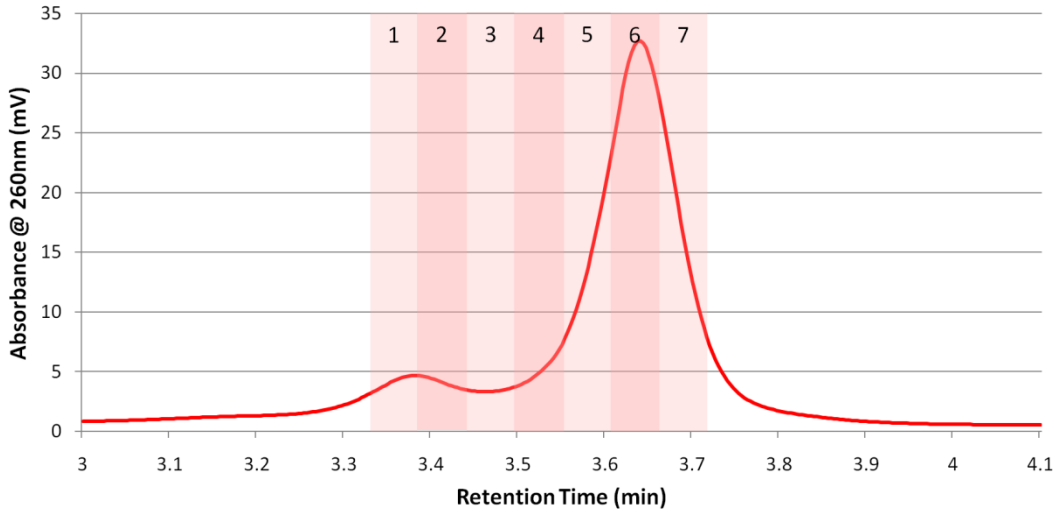


Figure 7.3: Analysis of sample CS-49 by TMHA, showing separation and capture of a single broad homoduplex peak (fractions 4-7). This major peak is preceded by a separate single heteroduplex peak (fractions 1-2). Based on analyses of the sequencing electropherograms, the greatest shift in peak height ratios was between fractions 4 and 7. LPA of these fractions made it possible to determine the haplotypes of the individual contributors to this mixture with an average basecalling confidence of 98.557%.

Of the samples displayed in table 7.1, those displaying two, three and four peak resolution by DHPLC account for 29.4%, 21.6% and 47.1% of the samples tested. Comparison to a larger study of 849 mixtures subjected to DHPLC profiling across all four forensically relevant regions of the human mtDNA (Chapter 2) reveals a slight decrease in the amount of samples yielding two and three peak resolution. In that study, samples displaying two, three and four peak resolution comprised 50.3%, 36.6% and 11.6% of all samples tested, respectively. This discrepancy may reflect differences in the specific sequences analyzed and the number of each sequence analyzed. In the current validation study, sequences were selected to incorporate a broad diversity of mixed base positions across the HV1 and HV2 regions. The larger study included numerous more similar amplicons which may be less likely to yield cross hybridization products capable of being fractionated into four clearly discernible chromatographic peaks.

§ 7 - 3.2 Efficiency of Component Identification as a Function of Mixture Ratio

Comparison of multiple mixture ratios for a two-component mixture yielded information on which fraction comparisons generate the most informative and consistent coordinated shifts in peak height fluorescence. Analysis of samples CS-8, CS-20, CS-21, CS-38 and CS-39 sequenced with the C1 primer and samples CS-15, CS-25, CS-26, CS-42 and CS-43 sequenced with the D2

primer made it possible to determine which fraction comparisons yielded the largest and the smallest coordinated shifts in the fluorescence ratios of linked bases. In all cases, the homoduplicies formed during cross hybridization of the amplified PCR products resolve into two distinct peaks, each representing one contributor. If the first peak is the tallest, the greatest coordinated shift in peak height fluorescence is achieved by comparing the lagging edge of the second peak to the top of the first peak (Figure 7.1A; fractions 7 and 11). If the second peak is the tallest, the same can be achieved by comparing the leading edge of the first peak to the top of the second peak. As described above, comparison of adjacent fractions tends to yield only limited shifts in peak height fluorescence ratios due to the fact that adjacent fractions are likely to be characterized by smaller changes in the underlying molar ratios of one contributor to the other.

When homoduplices are not resolved into separate peaks, comparisons between the heteroduplex peaks and the top or lagging edge of the homoduplex peak generally yields the greatest coordinated shift in peak height fluorescence. This can be illustrated when comparing fractions sequenced for CS-29 (Figure 7.4; fractions 1-3 compared to fractions 11-13). Here, a large shift in peak height fluorescence ratio was consistently observed when comparing a number of fractions on the lagging side of the heteroduplex peak to the top or lagging side of the homoduplex peak. When this is done, a consistent 20% - 30% coordinated shift is noted for all fraction comparisons performed between the homo- and

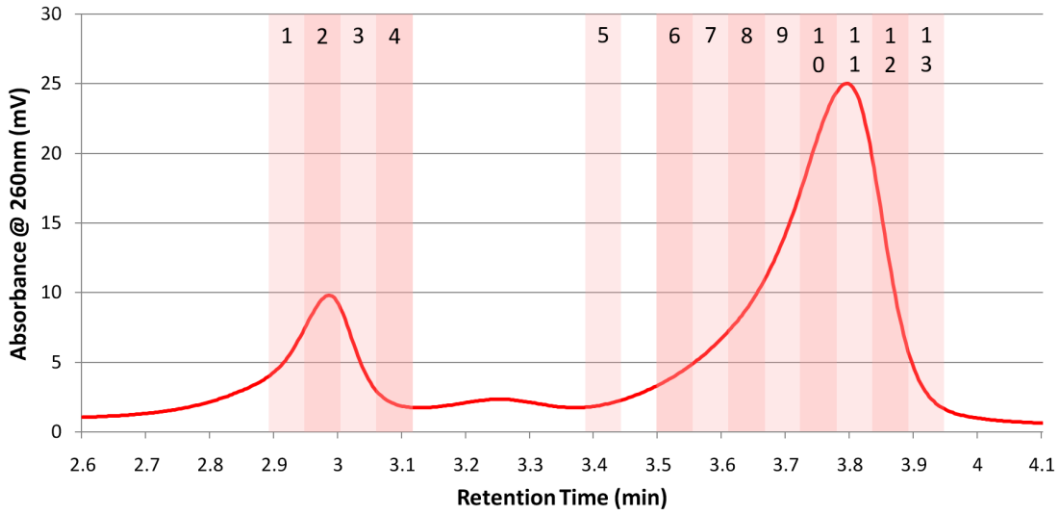


Figure 7.4: Analysis of sample CS-19 by TMHA, showing separation and capture of a single very broad homoduplex peak (fractions 6-13) preceded by a separate single heteroduplex peak (fractions 1-3). A consistent 26% - 32% coordinated shift in peak height fluorescence ratio is observed when comparing fractions on the lagging side of the heteroduplex peak (fractions 2 or 3) to the homoduplex peak (fractions 7-13). Based on analyses of the sequencing electropherograms, the greatest shift in peak height ratios was between fractions 2 and 8. LPA of these fractions made it possible to determine the haplotypes of the individual contributors to this mixture with an average basecalling confidence of 99.982%.

heteroduplex peaks. Only when fluorescence ratios were compared between fractions from the same peak was there a low shift in peak height fluorescence ratios.

§ 7 - 3.3 Body Fluid Mixtures

Thirty six two-component body fluid mixtures were stained onto a variety of substrates intended to simulate forensic casework. These included denim, fabric, leather, wood, synthetic carpeting, nylon and wallboard. Samples were aged at room temperature for four weeks followed by DNA extraction. No two donor samples used in this study had identical mtDNA haplotypes as previously determined by sequencing analysis of known buccal swabs (Table 7.2). Amplification of HV1 and HV2 region fragments was performed on a subset of questioned mixtures using forensically validated primer sets and conditions. All amplified samples were then fractionated by DHPLC under partially denaturing conditions. Five to nine fractions for each sample were analyzed by direct sequencing, followed by LPA of mixed base locations. DNA sequencing yielded clean base calls for all but mixed nucleotide positions, which were characterized by overlapping fluorescent peaks. Identification by LPA of the haplotypes of the individual contributors that comprised each of the 18 mixtures tested was achieved in all cases for a success rate of 100%. All identified haplotypes were in full concordance with sequence data obtained from sequencing of single source representative samples from each contributor. The linkage phase of individual

Table 7.2: The mtDNA control region sequences of the body fluid samples used in the current study. Sequences are shown as deviations from the revised Cambridge Reference Sequence (rCRS).

	Nucleotide Position (rCRS)																						
	HV1A				HV1B								HV2A			HV2B							
Sample	16069	16126	16144	16146	16189	16223	16270	16304	16311	16320	16342	16362	73	150	152	195	228	242	263	282	295	309.1	315.1
rCRS	**C**	**T**	**T**	**A**	**T**	**C**	**C**	**T**	**T**	**C**	**T**	**T**	**A**	**C**	**T**	**T**	**G**	**C**	**A**	**T**	**C**	**:**	**:**
1	·	·	·	·	·	·	·	C	·	T	·	·	·	·	C	·	·	·	G	·	·	C	C
2	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	G	·	·	·	C
3	·	·	·	·	·	T	·	·	·	·	·	C	G	·	·	·	·	·	G	·	·	·	C
4	·	·	C	·	C	·	T	·	·	·	·	·	G	T	·	·	·	·	G	·	·	·	C
5	T	C	·	·	·	·	·	·	·	·	·	·	G	·	·	C	A	·	G	·	T		C
6	·	·	·	G	·	·	·	·	·	·	C	·	G	·	·	·	·	·	G	C	·	C	C

contributors was determined with a high degree of base calling confidence. Depending on substrate and the nucleotide position, the mean confidence of base calling by linkage phase analysis ranged from an average of 99.926% to 99.994% (Table 7.3). In all cases, linkage phase analysis was performed on those DHPLC fractions which showed the greatest shift in peak height fluorescence ratios.

Comparison of all fractions sequenced yielded an average change in peak height fluorescence ratio ranging from 7.7% - 24.5%, with fractions neighboring each other yielding the lowest shift in peak height fluorescence (0.2% - 2.7%). The largest shift in peak height fluorescence ratio ranged from 16.3% - 51.5%. Those fraction comparisons that tended to yield the most optimal results were consistent with the aforementioned results of the studies in this chapter that examined the relationship between DHPLC trace characteristics and the efficiency of mixture resolution by DHPLC in combination with LPA.

§ 7 - 3.4 Environmental Insult Mixtures

Thirty six two-component body fluid mixtures were subjected to a variety of environmental insults designed to simulate forensic casework type samples. These included gasoline, soil, laundry detergent, used motor oil, sodium hydroxide and acetic acid. Following DNA extraction, amplification of mtDNA HV1 and HV2 regions was performed on a subset of mixtures using forensically

Table 7.3: Casework-type mtDNA samples analyzed by DHPLC and LPA including changes in peak height ratios attained and base calling confidence ranges for linkage phase determination at mixed nucleotide positions.

								All Sequenced Fractions		Maximum Δ Fluorescense		
Item	Substrate	DNA Source	Enrironmental Contaminant	Fragment	DHPLC peaks	Fractions Sequenced	Mixed sites detected	Ave Δ Fluorescence Range	Ave Δ Fluorescence	Δ Fluorescence Range	Confidence Ave.	Confidence Stdev.
1	Carpet	Blood/Saliva	None	HV1B	2	7	3	0.8% - 32.1%	16.3%	31.1% - 33.1%	99.983%	0.004%
2	Carpet	Saliva/Saliva	None	HV1B	4	9	4	1.3% - 26.5%	11.7%	19.2% - 30.4%	99.977%	0.014%
3	Carpet	Saliva/Semen	None	HV2A	2	5	3	0.7% - 28.7%	14.2%	19.9% - 33.8%	99.978%	0.019%
4	Carpet	Semen/Semen	None	HV1B	5	9	4+LH	0.8% - 40.2%	18.8%	28.4% - 51.5%	99.991%	0.004%
5	Denim	Blood/Blood	None	HV1B	5	9	4+LH	2.4% - 35.8%	17.0%	29.3% - 41.2%	99.987%	0.008%
6	Denim	Blood/Semen	None	HV2A	3	7	2	1.5% - 18.4%	7.7%	16.3% - 20.4%	99.926%	0.046%
7	Leather	Blood/Blood	None	HV1B	5	9	4+LH	1.5% - 38.9%	18.9%	29.1% - 51.0%	99.988%	0.008%
8	Leather	Blood/Saliva	None	HV1B	2	7	3	0.9% - 44.6%	24.5%	43.0% - 47.4%	99.994%	0.0002%
9	Leather	Blood/Semen	None	HV2A	3	7	2	0.6% - 21.5%	8.4%	16.8% - 26.1%	99.934%	0.057%
10	Leather	Saliva/Saliva	None	HV1B	4	9	4	0.4% - 26.6%	11.7%	17.4% - 31.8%	99.974%	0.021%
11	Nylon	Semen/Semen	None	HV1B	5	8	4+LH	0.2% - 40.7%	19.7%	31.6% - 46.9%	99.989%	0.006%
12	Nylon	Saliva/Semen	None	HV2A	2	6	3	0.7% - 27.8%	12.5%	19.2% - 32.6%	99.976%	0.018%
13	Wallboard	Blood/Saliva	None	HV1B	2	7	3	0.6% - 42.8%	23.1%	42.3% - 43.5%	99.993%	0.001%
14	Wallboard	Saliva/Saliva	None	HV1B	4	9	4	0.3% - 25.2%	11.6%	21.4% - 27.0%	99.974%	0.002%
15	Wood	Blood/Blood	None	HV1B	5	7	4+LH	2.7% - 35.0%	17.4%	28.4% - 40.7%	99.988%	0.002%
16	Wood	Blood/Saliva	None	HV1B	2	7	3	1.0% - 42.1%	23.2%	39.8% - 46.1%	99.991%	0.003%
17	Wood	Blood/Semen	None	HV2A	3	7	2	0.3% - 25.5%	10.0%	19.3% - 31.8%	99.969%	0.018%
18	Wood	Saliva/Saliva	None	HV1B	4	9	4	0.7% - 23.6%	10.4%	17.2% - 27.5%	99.965%	0.016%
19	Cotton Swab	Blood/Blood	Detergent	HV1B	5	8	4+LH	1.3% - 37.8%	19.7%	28.8% - 45.8%	99.991%	0.003%
20	Cotton Swab	Blood/Saliva	Detergent	HV1B	2	7	3	0.6% - 39.3%	21.4%	37.1% - 41.5%	99.992%	0.002%
21	Cotton Swab	Blood/Semen	Detergent	HV2A	3	6	2	0.8% - 22.1%	9.4%	17.0% - 27.1%	99.959%	0.024%
22	Cotton Swab	Blood/Blood	Gasoline	HV1B	5	8	4+LH	0.6% - 37.5%	19.7%	29.0% - 46.7%	99.986%	0.007%
23	Cotton Swab	Blood/Semen	Gasoline	HV2A	3	7	2	0.6% - 20.8%	8.3%	17.3% - 24.3%	99.958%	0.021%
24	Cotton Swab	Saliva/Saliva	Gasoline	HV1B	4	9	4	1.5% - 27.4%	12.1%	20.2% - 33.0%	99.975%	0.012%
25	Cotton Swab	Saliva/Semen	Gasoline	HV2A	2	6	3	0.7% - 18.4%	8.2%	12.2% - 21.7%	99.926%	0.079%
26	Cotton Swab	Blood/Blood	HAC	HV1B	5	7	4+LH	0.8% - 39.5%	21.8%	27.7% - 49.4%	99.989%	0.009%
27	Cotton Swab	Blood/Saliva	HAC	HV1B	2	7	3	0.5% - 41.9%	23.1%	39.4% - 46.1%	99.991%	0.003%
28	Cotton Swab	Saliva/Saliva	HAC	HV1B	4	8	4	1.1% - 26.9%	12.6%	19.1% - 30.4%	99.973%	0.012%
29	Cotton Swab	Blood/Blood	Motor oil	HV1B	5	9	4+LH	1.5% - 41.1%	19.2%	33.0% - 49.9%	99.991%	0.004%
30	Cotton Swab	Blood/Saliva	Motor oil	HV1B	2	7	3	0.3% - 43.4%	23.2%	41.5% - 47.2%	99.993%	0.001%
31	Cotton Swab	Saliva/Saliva	Motor oil	HV1B	4	9	4	1.6% - 28.4%	12.1%	24.7% - 33.3%	99.978%	0.007%
32	Cotton Swab	Blood/Semen	NaOH	HV2A	3	6	2	0.7% - 19.8%	9.0%	17.6% - 22.1%	99.954%	0.016%
33	Cotton Swab	Saliva/Semen	NaOH	HV2A	2	5	3	1.7% - 26.5%	12.8%	24.8% - 28.1%	99.978%	0.007%
34	Cotton Swab	Semen/Semen	NaOH	HV1B	4	6	4+LH	3.2% - 38.6%	16.4%	31.0% - 48.9%	99.987%	0.005%
35	Cotton Swab	Blood/Semen	Soil	HV2A	3	6	2	0.5% - 20.8%	8.0%	15.6% - 26.1%	99.929%	0.065%
36	Cotton Swab	Semen/Semen	Soil	HV1B	5	8	4+LH	0.9% - 21.5%	9.9%	20.4% - 23.0%	99.964%	0.006%
37	None	Bone	Aged	HV2A	4	6	5	0.7% - 54.6%	20.3%	48.7% - 61.6%	99.996%	0.001%
38	None	Bone	Aged	HV2A	3	6	5	0.3% - 21.6%	13.0%	11.2% - 39.1%	99.912%	0.088%
39	None	Axial hair/Blood	None	HV1A	3	7	2	0.2% - 19.0%	8.2%	13.7% - 24.3%	99.923%	0.070%
40	None	Head hair/Blood	None	HV1A	5	8	2+LH	1.5% - 32.3%	11.9%	31.3% - 33.4%	99.984%	0.004%
41	None	Head hair/Saliva	None	HV2B	2	5	2	1.1% - 13.4%	5.2%	13.0% - 13.9%	99.873%	0.000%
42	None	Head hair/Semen	None	HV1A	2	5	2	2.5% - 17.4%	9.0%	16.1% - 18.7%	99.919%	0.037%
43	None	Head hair/Semen	None	HV1A	2	5	4	1.9% - 25.0%	10.8%	22.6% - 28.0%	99.974%	0.009%
44	None	Head hair/Semen	None	HV2B	3	6	3	0.8% - 34.9%	18.5%	27.3% - 44.5%	99.987%	0.009%
45	None	Head hair/Blood	Dyed/None	HV1A	5	6	2+LH	1.0% - 18.6%	8.7%	15.6% - 21.7%	99.927%	0.062%
46	None	Head hair/Blood	Permed/None	HV1A	4	5	3+LH	2.0% - 13.2%	6.6%	11.0% - 16.1%	99.835%	0.058%
47	None	Pubic hair/Semen	None	HV1A	5	8	2+LH	0.9% - 29.8%	11.4%	28.8% - 30.7%	99.983%	0.004%
48	Cotton Swab	Saliva/Breat swab	None	HV1A	3	6	2	0.3% - 7.8%	3.7%	6.8% - 8.7%	99.493%	0.262%
49	Cotton Swab	Saliva/Breat swab	None	HV2B	3	7	3	1.3% - 15.2%	7.4%	5.7% - 25.8%	99.534%	0.672%
50	Cotton Swab	Vaginal swab/Semen	None	HV1A	3	6	2	0.2% - 16.2%	7.1%	14.5% - 17.9%	99.906%	0.052%

validated primer sets and amplification conditions. All amplified samples were fractionated by DHPLC under partially denaturing conditions. Five to nine fractions for each sample were then analyzed by direct sequencing, followed by LPA of all mixed base positions.

DNA sequencing yielded good quality base calls for all but mixed nucleotides positions which, as anticipated, were characterized by overlapping fluorescent peaks. For each of the 18 amplified mixtures, the haplotypes of the individual contributors was determined by LPA of the sequence electropherograms. All identified haplotypes were in full concordance with sequencing results obtained from single source reference samples of the same contributors. Thus a success rate of 100% was achieved for the resolution of the 18 mixtures that were fractionated. Moreover, the average base calling confidence (depending on substrate and nucleotide position) ranged from 99.926% to 99.993% (Table 7.3). This was achieved when the DHPLC fractions that were compared were those that displayed the greatest shifts in peak height fluorescence.

Pairwise comparisons of all fractions sequenced yielded an average change in peak height fluorescence ratio across all mixed base positions ranging from 8.0% - 23.2%. As expected and as demonstrated previously, adjoining fractions yielded the lowest shift in peak height fluorescence ratios (0.3% - 1.7%).

The largest shifts in peak height fluorescence ratios ranged from 12.2% - 49.9%. Those fraction comparisons that tended to yield the most optimal results were consistent with the aforementioned results of the studies in this chapter that examined the relationship between DHPLC trace characteristics and the efficiency of mixture resolution by DHPLC in combination with LPA.

§ 7 - 3.5 Hair and Bone Mixtures

Hair shafts as evidentiary material are commonly encountered in forensic contexts. Because of the paucity of nuclear DNA typically recovered from these samples mtDNA analysis often represents an investigator's most promising opportunity to obtain potentially probative biological information. Accordingly, 31 hair samples were contaminated with various body fluids. Head, axial and pubic hairs were immersed in blood, semen and saliva and allowed to air dry. DNA was extracted from all samples, yielding a total of 9 hair shaft body fluid mixtures which were detected and subsequently fractionated by DHPLC. Sequence data from the recovered DNA fractions were subjected to LPA in an effort to determine the haplotypes of the individual contributors to the mixtures. For each of the 9 mixtures that were fractionated and analyzed, LPA correctly identified the contributor haplotypes in 100% of the cases. All identified haplotypes were in full concordance with sequencing results obtained from single source reference samples of the same contributors. Haplotypes were determined with a high base calling confidence which (depending on the specific

mixture and nucleotide position) ranged from an average of 99.84% to 99.99% (Table 7.3). This was achieved when the DHPLC fractions that were compared were those that displayed the greatest shifts in peak height fluorescence.

Pairwise comparison of all fractions sequenced yielded an average change in peak height fluorescence ratio ranging from 5.2% - 18.5%. As before, adjoining fractions yielded the lowest shift in peak height fluorescence (0.2% - 2.5%). The largest shifts in peak height fluorescence ratios ranged from 11.0% - 44.5%. As has been seen with all other mixed samples, the fraction comparisons that tended to yield the most optimal results were consistent with the aforementioned results of the studies in this chapter that examined the relationship between DHPLC trace characteristics and the efficiency of mixture resolution by DHPLC in combination with LPA.

Human bone samples obtained from the University of Montana's Department of Anthropology were pulverized and then processed to extract amplifiable mtDNA. One bone sample yielded no amplifiable product even following a second round of extraction and amplification, while two of the samples yielded a mixed mtDNA profile. The lack of amplifiable product for one of the bone samples was most likely due to a number of factors. These may include but are not limited to extensive degradation of the DNA present in the

bones which had been stored for years at room temperature and/or chemical treatments used to prepare the material for storage.

A second bone (Item-37) did yield amplifiable mtDNA. As is typical, the porous nature of many aged bone samples makes them conducive to contamination by extraneous DNA, *e.g.*, from comingling with other remains or excessive handling by unrelated individuals. Initial analysis of the amplified mtDNA by DHPLC under partially denaturing conditions suggested that the amplified product consisted of a mixture of two non-identical amplicons. Fractionation of this sample by DHPLC yielded six fractions from which DNA could be recovered and sequenced. Pairwise comparisons of the resulting sequence data from these fractions revealed a two-component mixture with five mixed-nucleotide positions. The largest shift in the peak height ratios among the fractions compared was 48.7% - 61.6% depending on the mixed nucleotide position being examined. LPA of the sequence data from these fractions resulted in an average base calling confidence of greater than 99.99% across all five mixed base positions.

A third bone (Item-38), also presented as a likely two-component mixture upon examination of the DHPLC trace characteristics under partially denaturing conditions. Fractionation of this sample by DHPLC yielded six fractions from which DNA could be recovered and sequenced. Shifts in peak height fluorescence

ratios that were evident in pairwise comparisons of sequence data from these six fractions revealed a total of five mixed-base positions. Subsequent LPA, of the mixed-base positions however, revealed that while shifts in peak height ratios made it possible to determine the linkage phase of one contributor, there was not a corresponding linkage phase at these same positions for the second contributor. Rather, coordinated shifts in peak height ratios at only two of the five mixed nucleotide sites appeared to constitute the linkage phase of a second contributor (average base calling confidence >99.9%). Shifts in peak height ratios at the remaining three mixed base position appeared to constitute the linkage phase of a third contributor (average base calling confidence >99.9%). While the third component was not identified in the initial screen by DHPLC, this was most likely due to the low concentration of the third contributor in this mixture. The presence of the third contributor, however, was evident in the DNA sequencing electropherograms and was resolved through LPA.

Although a three-contributor mixture was discovered and resolved for this particular sample during LPA, extensive studies on three or more component mixtures at varying contributor ratios remain a significant challenge. This is due to the difficulty that is typically encountered in attempting to deconvolute mixed nucleotide positions where all three contributors differ (Chapter 6).

Neither chromatographic trace characteristics nor the number of variant positions between the amplicons comprising a mixture revealed any significant trends in the efficiency of separation for any of the samples tested in this study. In fact, two-peak DHPLC traces yielded both the lowest and greatest averages with regard to the observed shifts in peak height ratios. It is recognized, however, that based on an inspection of the results reported from this study, mixtures of amplicons sequenced using the C1 primer tended to yield a lower shift in peak height ratios and therefore a lower efficiency of mixture separation. This trend, however, is most likely due to the specific mixtures employed in this study. This is supported by the results obtained for the control samples presented in Table 7.1 and data previously reported on concentration and sequencing reproducibility (Chapter 4) where this trend was not evident.

§ 7 - 4 Conclusion

Mitochondrial DNA sequencing analysis is now a widely used methodology for the identification of sequence polymorphisms both in the medical and forensic communities. While mtDNA sequencing analyses of samples containing single contributors have been rigorously tested and validated, analyses of the electrophoretic trace characteristics of mtDNA mixtures have, until now, never been thoroughly studied. Alternative methods for mixture separation (*e.g.*, DGGE, pyrosequencing) have previously been proposed as

means for separating mtDNA mixtures. Use of DGGE, however, is extremely labor intensive, requires significant sample manipulation, re-amplification of captured PCR products and is not easily amenable to automation (Danielson *et al.,* 2005). Pyrosequencing yields very short sequence reads (~70bp) and consumes significant amounts of sample due to multiple synthesis reactions. Further, mixture separation capabilities of pyrosequencing have yet to be put through the rigors of forensic validation and pyrosequencers remain more of a research novelty than a realistic option for forensic practitioners. The significant cost of the requisite instrumentation presents an additional barrier.

Although mtDNA sample contamination by even minor extraneous sources of human DNA remains high due to its environmental persistence and abundance in terms of copy number, proper handling procedures and vigorous quality control measures can significantly reduce the occurrence of cross contamination. In the current study, no mixture component was traced back to individuals not originally added to the mixture. Not even in the case of Item-38, was the third component traced back to any individual present in the laboratory's internal exclusion database. This database includes not only all laboratory employees, but also all human DNA samples located in the laboratory.

In the current study, the analysis of mtDNA mixtures from two-contributor samples was evaluated using DHPLC fractionation and LPA of

sequencing results obtained from captured fractions. Using this approach, a total of 101 mixtures have been analyzed and the haplotypes determined for individual contributors to each mixture. In all cases, linkage phases at mixed-base positions were determined with a high degree of base calling confidence. Nucleotides determined to be in linkage phase with each other were, in all cases, fully concordant with sequencing results obtained from non-mixed reference samples. Results from the current study therefore, demonstrate the usefulness and reliability of this approach through the use of both control and simulated casework samples.

§ 7 - 5 Summary

Mixtures of two or more contributors decrease the reliability and usefulness of mtDNA haplotyping, due to the unavoidable generation of ambiguous and often uninformative sequencing data. Fractionation and recovery of PCR amplicons by DHPLC has made it possible to separate and identify individual contributors to a mixture without reamplification or excessive processing of PCR products. Statistical analysis using linkage phase analysis adds statistical weight to separated mixtures allowing for the confidence of mixed nucleotide separation to be determined. This study presents results of mixture resolution by DHPLC in combination with LPA using a variety of commonly encountered and environmentally insulted samples as well as hair, bone etc.

designed to simulate evidentiary material consistent with forensic casework. These validation studies have been designed to facilitate the adoption of this technology by forensic practitioners. The current study shows that mtDNA mixture fractionation by DHPLC and sequence analysis by LPA is a rapid, highly reliable and forensically valid method for mtDNA mixture separation and subsequent haplotype determination of individual contributors to a mixed sample.

Chapter 8: Validation of Denaturing High-Performance Liquid Chromatography for Forensic mtDNA Casework Analysis

§ 8 - 1 Introduction

Denaturing high-performance liquid chromatography (DHPLC) is a rapid ion-paired reversed-phase HPLC method used to detect variations in DNA sequence in identical length amplicons. The use of DHPLC has in recent years been proposed as a method for a variety of forensic applications. These methods include but are not limited to detection of single nucleotide polymorphisms (Shi *et al.,* 2001), sex identification (Shinka *et al.,* 2001), heteroplasmy determination (van Den Bosch *et al.,* 2000; Biggin *et al.,* 2005), short tandem repeat (STR) analysis (Zhu *et al.,* 2006) and mtDNA screening and mixture resolution (Danielson *et al.,* 2007).

In DHPLC, cross-hybridized PCR products interact with the hydrophobic column matrix through a triethylammonium acetate buffer and are eluted through an increasing gradient of acetonitrile (Huber *et al.,* 1993). Under partially denaturing conditions less stable heteroduplices elute prior to the more stable homoduplices allowing for identification of sequence variation (O'Donovan *et al.,* 1998; Hou and Zhang 2000). Under non-denaturing conditions, samples of varying length are easily separated, allowing for sample purification and

determination of amplicon size and quantity in a similar manner as other HPLC systems (Warren and Doniger 1991; Henninger *et al.,* 1993; Zeillinger *et al.,* 1993; Katz 1996).

While new protocols for analysis and processing of evidentiary material employing DHPLC analysis have been reported (Danielson *et al.,* 2003; Danielson *et al.,* 2005; Danielson *et al.,* 2007), forensic validation studies of the WAVE® 3500HT DNA Fragment Analysis System (Transgenomic, Inc., Omaha, NE) employed for DHPLC analysis have until now not been carried out. This has limited DHPLC use to non-caseworking applications. The aim of the current study was, therefore, to demonstrate the reliability, sensitivity and consistency of the WAVE® 3500HT DNA Fragment Analysis System in combination with the DNASep® column (Transgenomic, Inc.). The results of this study demonstrate that the combination of WAVE® 3500HT DNA Fragment Analysis System and DNASep® column provides a platform for consistent and reliable analysis of cross-hybridized PCR products with unparalleled detection sensitivity.

§ 8 - 2 Materials and Methods

§ 8 - 2.1 Mitochondrial DNA Extraction

All aspects of this research were conducted in compliance with U.S. Federal Policy for the Protection of Human Subjects (56 FR 28003). Individual buccal samples were collected from unrelated subjects who had previously

provided informed consent to participate in the study. Total human DNA was extracted using the EZ1 DNA tissue kit on the Qiagen BioRobot EZ1 DNA extraction robot (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. Samples were eluted into 200µl of nuclease-free water and then stored at -20°C until PCR amplification.

§ 8 - 2.2 Mitochondrial DNA Control Region Amplification

Forensically validated protocols (Wilson *et al.,* 1995; LaBerge *et al.,* 2003) were used to amplify the four forensically relevant regions (HV1A: 278bp; HV1B: 271bp; HV2A: 278bp; HV2B: 277bp) of the human mitochondrial genome. Amplification reactions (50µL) were prepared using 50pmol each of forensically-validated PCR primers at final concentration of 1µmol/L (Wilson *et al.,* 1995; LaBerge *et al.,* 2003); 0.45 U/10µL AmpliTaq GOLD® DNA polymerase (Applied Biosystems, Foster City, CA); 0.05 U/10µL PFU DNA polymerase (Stratagene, La Jolla, CA); 10X AmpliTaq GOLD® Buffer (Applied Biosystems) and 200µmol/L of each dNTP (Stratagene) with 2µL of an approximately 10pg/µL DNA extract. Amplifications were carried out on a GeneAmp® 9700 thermocycler (Applied Biosystems) with an initial denaturation at 95˚C for 10 minutes, followed by 32 cycles of 95˚C for 20 seconds, 60˚C for 30 seconds and 72˚C for 45 seconds, followed by a final extension at 72˚C for 15 minutes.

§ 8 - 2.3 DHPLC-Based Temperature-Modulated Heteroduplex analysis (TMHA)

Amplified fragments were combined at a 1:1 molar ratio to generate a series of pair-wise mixtures which were cross-hybridized by denaturation at 95°C for 4 minutes and gradual cooling (1.5°C/min) to a final temperature of 25°C (LaBerge *et al.,* 2003). Cross-hybridized amplification reactions were assayed by DHPLC (Kuklin *et al.,* 1997) on a WAVE® 3500HT DNA Fragment Analysis System (Transgenomic Inc., Omaha, NE) containing a DNASep® analytical column (Huber *et al.,* 1993). Products were bound to the resin in an aqueous buffer containing 0.1M triethylammonium acetate (TEAA) pH 7.0 as an ion-pairing reagent. Cross-hybridized mixtures were analyzed under partially denaturing conditions at the empirically determined optimal temperatures for each of the four mtDNA amplicons (*i.e.*, HV1A, 58°C; HV1B, 59.2°C; HV2A, 56.5°C and HV2B, 57°C). All samples were eluted at 0.9ml/min flow rate and detected by UV absorbance (260nm). The optimal acetonitrile linear gradient generated from differential mixing of buffer A (0.1M TEAA) and buffer B (0.1M TEAA, 25% ACN) were 56% to 65% buffer B increase in 3.5 minutes for HV1A, HV2A and HV2B and a 55% to 64% buffer B increase in 3.5 minutes for HV1B. Eluted peaks were captured by automated fraction collection and then evaporated by vaccum centrifugation using a Centrivap Mobile Concentrator System (Labconco Corp, Kansas City, MO). Each sample was then resuspended in ddH_2O and stored at -20°C until assayed.

§ 8 - 2.4 Mitochondrial DNA Subcloning and DHPLC Analysis

Primers mt-8294F (5'-CCACTGTAAAGCTAACTTAGCATTAACC-3') and mt-8436R (5'- CCATACTCCTTACACTATTCCTCATCAC-3') were used to amplify a 143bp mtDNA coding region fragment (Andreasson *et al.,* 2002). The resulting amplification products were purified using Wizard® PCR Preps (Promega, Madison, WI), ligated into the pGEM®-T vector (Promega) and electroporated into DH5α electrocompetent cells. Cells were selected on LB agar plates containing 50μg/ml ampicillin and screened by PCR to identify plasmids containing target inserts. Plasmids were isolated from overnight cultures of these colonies by alkaline lysis and capture on glass-filter binding plates (Millipore, Bedford, MA). The aforementioned primers were used to amplify the 143bp fragment from isolated plasmids using conditions described above.

Amplification reactions were assayed by DHPLC under non-denaturing conditions (50°C) and differential mixing of buffer A and buffer B, where buffer B increased from 49.3% to 58.3% in 4.5 minutes. Samples were detected by UV absorbance (260nm) and fluorescence (ex. 490nm, em. 520nm). Fluorescence detection was made possible by employing a proprietary DNA intercalating dye (Transgenomic Inc.), injected after the eluent had passed the UV detector using an inline high sensitivity accessory (HSX) pump (Transgenomic, Inc.). Eluted peaks were captured by automated fraction collection and then evaporated by vaccum centrifugation.

§ 8 - 2.5 Dye-Terminator Sequencing

Dye-terminator sequencing was performed using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Samples were prepared in 10µL reaction volumes according to the manufacturer's protocols using the maximum available DNA input quantity up to a maximum of 0.8ng. Dye-terminated products were purified on Performa® DTR V3 96-Well Short Plates (Edge Biosystems, Gaithersburg, MD) and resolved on a PRISM® 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocols using the POP-6™ polymer and 47cm x 50µm capillaries (Applied Biosystems). The raw electrophoretic traces were analyzed using the KB Basecaller (Applied Biosystems) together with the appropriate dye set mobility file for the v1.1 kit using Sequencing Analysis Software v.5.1.1 (Applied Biosystems). The resulting sequence data were aligned using the Sequencher™ v4.2 DNA analysis software (Gene Codes Corp, Ann Arbor, MI).

Appropriate positive and negative controls were carried through the entire sample handling process to enable the detection of any cross contamination and ensure proper dye-terminator incorporation efficiency.

§ 8 - 3 Results and Discussion

§ 8 - 3.1 Sample Cross Contamination

Sample cross contamination as a function of injection-to-injection carryover was determined by capture of zero-volume injections (i.e., DNA-free samples) following the injection of amplified PCR samples. In these assays, samples containing high quantities (approx. 550ng) of amplified 143bp DNA fragment (from previously subcloned mtDNA coding region) were injected onto the DNASep® column yielding a single peak with maximum absorbance at 3.82 ± 0.05 min. This was followed by 5 zero-volume injections. The column eluent (flow path buffer) was then collected for the same time frame where DNA from the initial injection had eluted (*i.e.* 3.0 - 4.5 min). Sample detection by UV absorbance revealed no evidence of DNA carryover in any of the zero-volume injections performed. However, the use of a DNA intercalating dye and detection by fluorescence revealed DNA carryover in the initial zero-volume injection, ranging from approx. 35pg - 125pg. Four subsequent zero-volume injections yielded approx. 17pg, 14pg, 9pg and 8pg, respectively, as determined by extrapolating data from a fluorescence quantitiation standard curve determined from DNA quantities ranging from 0.625ng - 200ng (R^2 = 0.995) (Chapter 3).

Eluent captured from the zero volume injections was evaporated by vaccum centrifugation and resuspended in 10µL of ddH_2O, followed by dye-terminator labeling of 2µL of the resuspended product. The dye-terminator

sequencing reactions from the captured time period yielded no detectable sequence (data not shown). It is worth noting, that although injection-to-injection carryover was seen in the current study, the amount of DNA injected onto the DNASep® column (550ng) was significantly higher than the quantity of amplified mtDNA from reference samples which typically range from 50ng - 200ng. When sample quantities are in this more casework-realistic range, no injection-to-injection carryover is observed by UV or fluorescence detection.

In routine DHPLC analysis of over 30000 injections of research and forensic validation samples, evidence of cross contamination between injected samples was not observed. Only in the case of column saturation, as reported above, has there been identifiable column carryover between repeat injections. In an effort to completely eliminate any potential for injection cross contamination, following injections of large quantities of PCR products, the column can be washed with 75% acetonitrile for 10-15min. This removes any spurious amplification products that might remain in the system.

Per the recommendation of the manufacturer (Transgenomic, Inc.) columns were regularly inspected for integrity of the column matrix by injecting a set of calibration standards. This ensures also that the oven, which the column is housed in, is performing according to manufacturers' specifications. Regularly each column was purged of additional amplification materials (*i.e.,* polymerase)

which bind to the anterior side of the column. This was done by reversing the column within the flow path and purging it with 100% ACN at 70°C.

§ 8 - 3.2 Amplification reproducibility

In an effort to demonstrate the reproducibility of DNA analysis by DHPLC, ten replicates of all four forensically relevant mtDNA amplicons were amplified using previously validated protocols. The resulting PCR products were cross-hybridized at equal contributor ratios, and sequentially assayed under partially denaturing conditions by DHPLC. The resulting data indicate that DHPLC resolution is highly reproducible for each of the tested amplicons. Both the maximum peak height (Figure 8.1) and retention time for each peak height maxima (Figure 8.2) were determined for the four forensically relevant amplicons. Assay-to-assay variability was found to be negligible among independent amplification reactions as evidenced by the small standard deviations for each peak among the replicates. Variability as determined by peak retention time for the 10 replicate amplification reactions ranged from ±0.2 to ±0.8%. Variability as determined by peak height across all replicate amplification reactions ranged from ±3.1 to ±14.1%. Differences in maximum peak height may, however, be attributed to variables other than DHPLC, such as the efficiency of the amplification reaction itself.

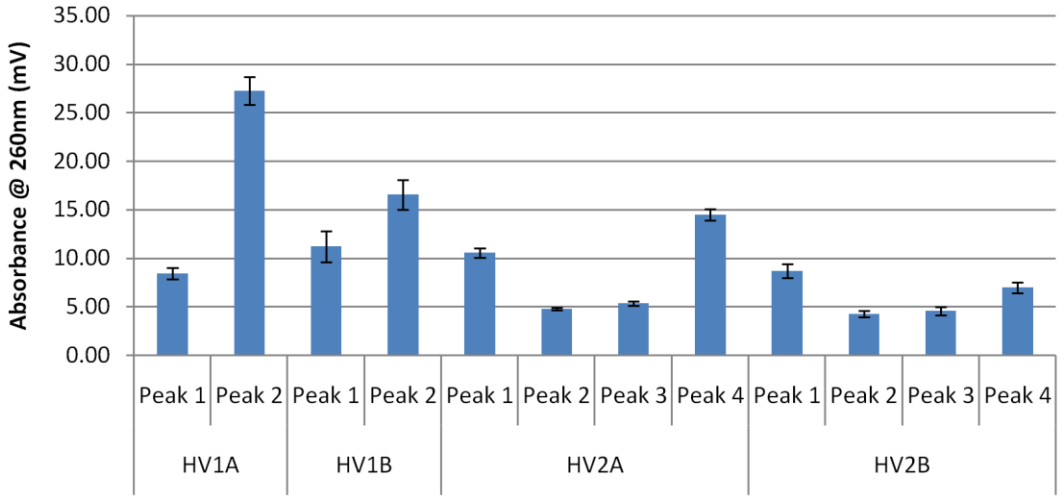


Figure 8.1: Peak heights (Mean ± SD) determined from chromatographic traces of representative 50:50 mixtures across all four forensically relevant amplicons under partially denaturing conditions. The results demonstrate the low level of assay-to-assay variability among ten independently replicated amplification reactions sequentially assayed by DHPLC.

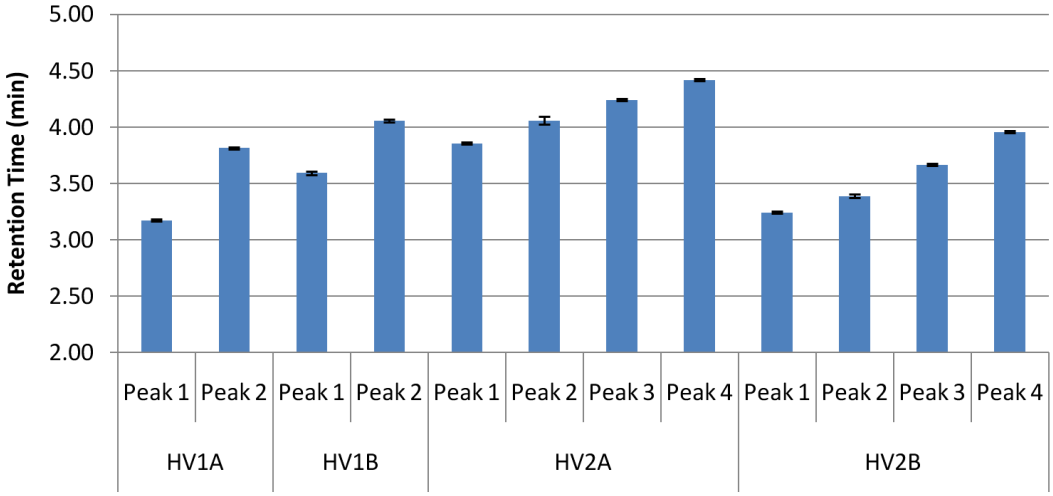


Figure 8.2: Retention times (Mean ± SD) determined from chromatographic traces of representative 50:50 mixtures across all four forensically relevant amplicons under partially denaturing conditions. The results demonstrate the low level of assay-to-assay variability among ten independently replicated amplification reactions sequentially assayed by DHPLC.

§ 8 - 3.3 Injection-to-injection reproducibility

To demonstrate the reproducibility of DHPLC for independent injections of identical DNA samples, four forensically relevant mtDNA amplicons were amplified (HV1A, HV1B, HV2A and HV2B). The resulting products were cross-hybridized at equal contributor ratios, and sequentially injected ten times under partially denaturing conditions by DHPLC. The resulting data indicate that sequential injections of cross-hybridized products are highly reproducible for each of the forensically relevant amplicons. Maximum peak heights (Figure 8.3) and peak retention times (Figure 8.4) were determined for each of the four amplicons. Assay-to-assay variability was found to be negligible among independent amplification reactions as evidenced by the small standard deviations for each peak among the replicates. Variability as determined by peak retention time for the 10 replicate amplification reactions ranged from ±0.1 to ±2.4%. Variability as determined by peak height across all replicate amplification reactions ranged from ±0.2 to ±1.8%.

§ 8 - 3.4 Column-to-column reproducibility

Reproducibility of DHPLC across multiple DHPLC DNASep® columns was determined using ten two-component cross-hybridized mixtures. The mixtures were sequentially injected onto ten different DNASep® columns and analyzed under partially denaturing conditions. The results show that DHPLC assay results are highly reproducible even when comparing across multiple DNASep® columns.

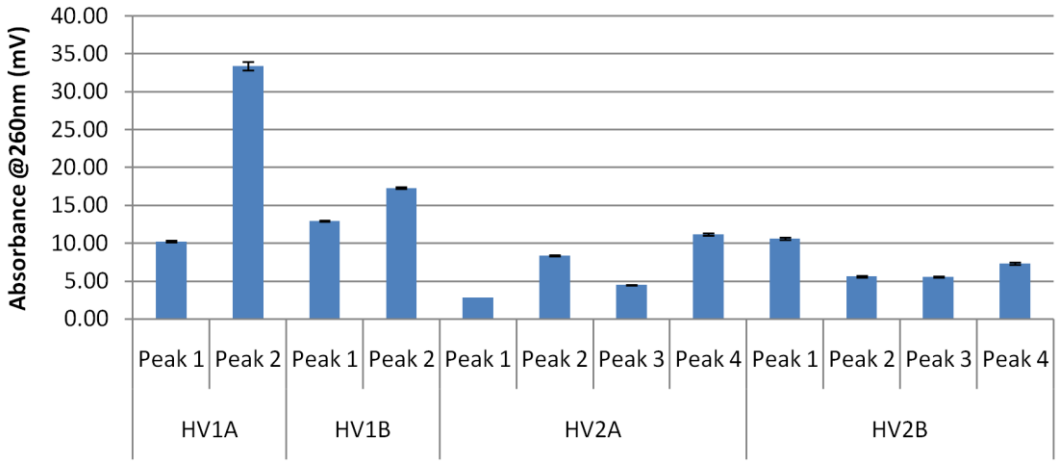


Figure 8.3: Peak heights (Mean ± SD) determined from chromatographic traces of representative 50:50 mixtures across all four forensically relevant amplicons under partially denaturing conditions. The results demonstrate the low level of assay-to-assay variability among ten replicate injections of a single amplification reaction assayed sequentially by DHPLC.

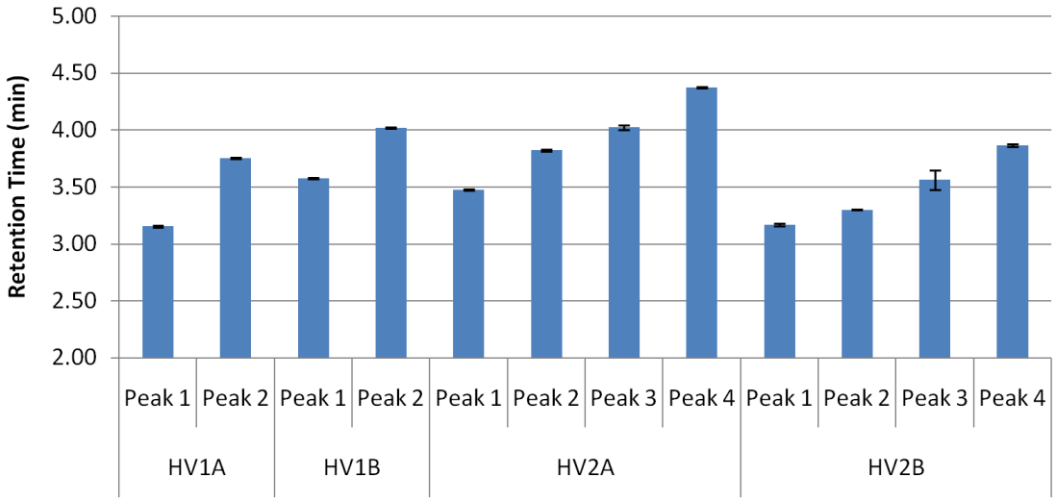


Figure 8.4: Retention times (Mean ± SD) determined from chromatographic traces of representative 50:50 mixtures across all four forensically relevant amplicons under partially denaturing conditions. The results demonstrate the low level of assay-to-assay variability among ten replicate injections of a single amplification reaction assayed sequentially by DHPLC.

Variability as determined by peak retention time for the replicate injections onto 10 individual DNASep® columns ranged from ±10.4 to ±23.5%. Although column-to-column retention times are relatively large compared to amplification and injection reproducibility, variability determined by comparison of relative retention times of each peak to any other peak detected in the mixture, was significantly smaller (Figure 8.5). Variability as determined by relative retention times of each peak to any other peak detected in the mixture ranged from ±1.5 to ±8.4%. Fluctuations in peak height absorbance were minimal although, in one case, the observed baseline was slightly above the mean for the other nine columns (data not shown). This was most likely an artifact of equilibration time as it disappeared with additional injections onto the column.

The DNASep® column is guaranteed by the manufacturer to last 6000 injections and can be routinely checked for peak resolution integrity. Qualitative assessment of DNASep® column integrity and resolution capabilities is performed by routine injection of column integrity standards. A number of compounds are known to have detrimental effects on DNASep® column performance. These include, but are not limited to bovine serum albumin (BSA), mineral oil, formamide, polyethylene glycol (PEG), glycerol, DMSO and a variety of detergents. Degradation of the column matrix can also occur as a result of improper storage, such as not storing it in 75% acetonitrile or letting the column

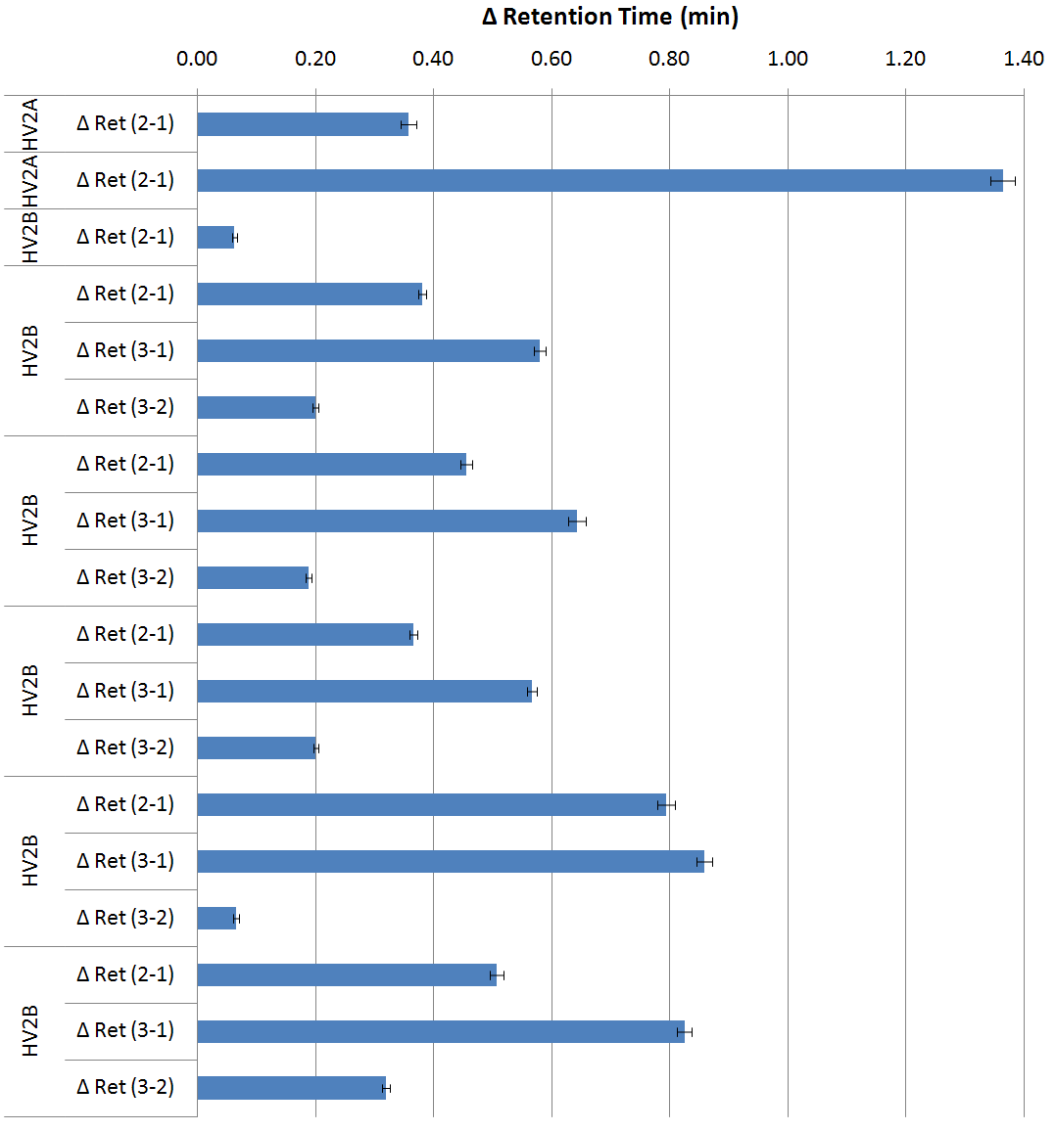


Figure 8.5: Average peak-to-peak spacing (Mean ± SD) determined from chromatographic traces of representative 50:50 mixtures across the two HV2 forensically relevant amplicons assayed on ten independent DNAsep® columns under partially denaturing conditions. The results demonstrate that when normalized on the basis of relative peak-to-peak retention times, column-to-column variability is minimized.

run dry. Degradation is observed by decreased resolution of standards peaks, given that oven temperatures are accurate.

§ 8 - 3.5 Negative and Reagent Blank Control Screening

Due to excessive time and sample handling cost associated with the sequencing of negative controls and reagent blanks in mtDNA casework analysis, HPLC represents an attractive alternative for the analysis of negative and reagent blank controls. UV and fluorescence detection limits using the DNASep® column have previously been determined and validated (Chapter 3, automated peak detection using the DHPLC Navigator™ Software: UV = 1.85±0.56ng; fluorescence = 30.9±1.4pg, manual peak detection: UV = approx. 0.5ng; fluorescence = <10pg). HPLC detection of the minimum amount of mtDNA amplicon required by dye-terminator sequencing was carried out by serial dilutions of purified HV2A mtDNA fragments at quantities frequently encountered by forensic practitioners. Replicate fragment quantities of 32.5pg, 62.5pg, 0.125ng, 0.25ng, 0.5ng, 1ng and 2ng were sequentially injected in triplicate onto the DNASep® column and analyzed using non-denaturing conditions. Injected amplicon quantities lower than 0.5ng showed no detectable deviation from baseline as observed by UV absorbance for any of the replicate injections (Figure 8.6). The slight absorbance increase observed over time in the UV baseline is a function of increase in acetonitrile concentration as the gradient gradually shifts from low to high concentrations of acetonitrile. All injected quantities of amplicon onto the

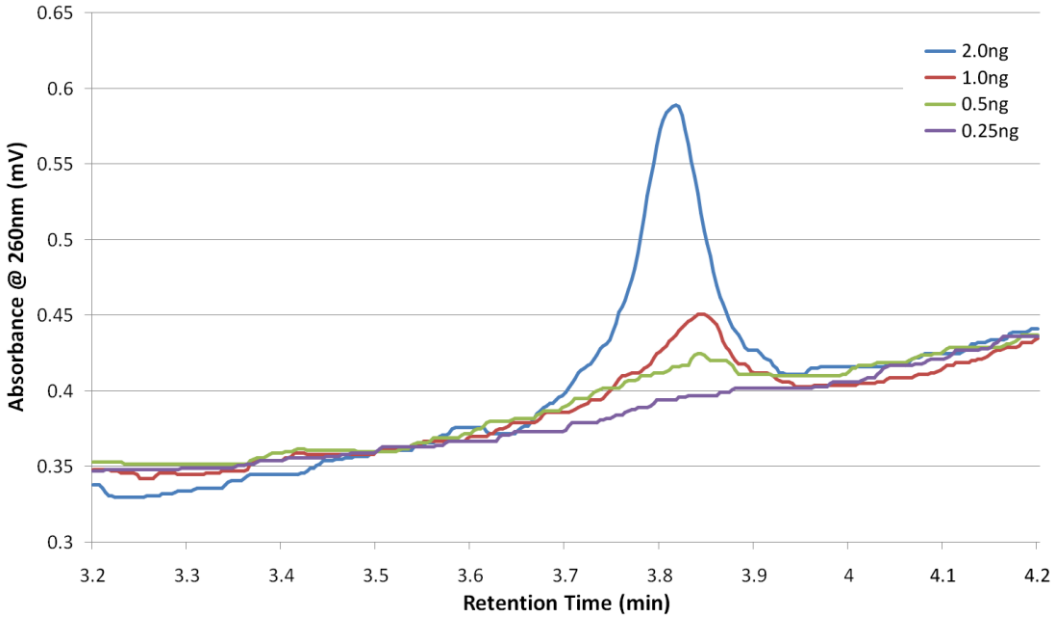


Figure 8.6: Representative layered chromatogram showing the lower limits of UV detection of a serially diluted DNA sample injected onto a DNASep® column. Injected amplicon quantities lower than 0.5ng showed no detectable deviation from baseline.

DNASep® column were detected by DNA intercalating-dye fluorescence detection (data not shown), consistent with results obtained for the cross-contamination study described above and results obtained in Chapter 3. All injected samples were also subjected to dye-terminator sequencing analysis for correlation purposes. Samples labeled with 2µL of 0.0125ng/µL (corresponding to the 62.5pg HPLC injection of 5µL of the same sample) repeatedly failed to yield any detectable sequence data (using 30sec dye-terminator sample injection times).

Based on these data, analysis of negative and reagent blank controls by HPLC can, therefore, be performed using fluorescence detection and the HSX inline accessory due to the superior detection capabilities. Given that quantities of amplificed product in a negative or reagent blank control don't exceed the 0.0125ng/µL needed to successfully obtain a detectable sequence there is no need to waste valuable time and reagents in confirming the absence of sequencing data.

§ 8 - 4 Summary

Denaturing High-Performance Liquid Chromatography (DHPLC) was validated as a tool for casework analysis of mtDNA amplicons. The WAVE® 3500HT DNA Fragment Analysis System in combination with the DNASep® column was evaluated based on its reliability, sensitivity and consistency of

analysis of cross-hybridized PCR products. Using subcloned mtDNA fragments, the WAVE® 3500HT DNA Fragment Analysis System was tested for run-to-run sample cross-contamination as detected by UV and fluorescence detection methods. Only at DNASep® column saturation was cross-contamination detected by high sensitivity fluorescence detection. Capture of run-to-run carryover products, however, yielded DNA quantities below that required for dye-terminator sequencing. Repeated amplification, injection-to-injection and column-to-column reproducibility were also determined by serial injections of mtDNA amplicons. Variability in peak height and peak retention times was negligible as evidenced by the small standard deviations recorded. Column-to-column variability was slightly higher, although comparison of relative retention times of each peak to any other peak detected in the mixture was negligible. HPLC screening of negative and reagent blank controls, routinely used in casework analysis, was evaluated as a means for reducing dye-terminator sequencing load in a forensic context. Negative or reagent blank controls yielding less than 0.0125ng/µL were confirmed as not yielding a detectable sequence data.

The results of this study demonstrate that the combination of WAVE® 3500HT DNA Fragment Analysis System and DNASep® column provides a platform for consistent and reliable analysis of cross-hybridized PCR products with unparalleled detection sensitivity. Additionally, the use of WAVE® 3500HT

DNA Fragment Analysis System and DNASep® column streamlines mtDNA analysis by combining sample purification, quantification and length-characterization into a single step.

Chapter 9: Streamlining Mitochondrial DNA Sequencing Analysis for Forensic Casework Samples

§ 9 - 1 Introduction

In recent years, the number of forensic evidentiary samples that are submitted for DNA analysis have increased dramatically. This is in part due to significant increase in knowledge of DNA capabilities in the law enforcement community and general acceptance of DNA evidence in court. Forensic scientists have, therefore, focused significant effort on not only developing new methods for retrieving and analyzing DNA evidence (*e.g.*, low copy number (LCN), severely degraded remains), but also streamline and enhance methods already in use by the forensic community. This has resulted in significant advances in automated robotics, sample handling methods and data analysis.

When traditional short tandem repeat (STR) DNA analyses fail, mitochondrial DNA (mtDNA) sequence analysis may provide investigators with useful information. This can help to advance criminal investigations (Allen *et al.*, 1994; Allen *et al.*, 1998) or give emotional closure to families by identifying the remains of loved ones (Holland *et al.*, 1993). Mitochondrial sequence analyses, however, do have limitations. Due to its strict maternal inheritance, lack of recombination and inability to identify single individuals, mtDNA analysis cannot

offer the kind of discrimination power that is seen when STR data are obtained. Furthermore, mixtures of mtDNA are frequently observed. These mixtures can exist within a single individual (*i.e.*, heteroplasmy) or as situational mixtures. This negatively impacts the value of mtDNA sequence analysis in two ways. First, it lowers the power of discrimination because mixed nucleotide positions must either be excluded from the reported results (*e.g.*, length heteroplasmy) or used in calculations of the number of possible haplotypes represented by the sequence data. Second, the observation of a mixture in the resulting sequence data is most typically reported as uninterpretible. A means of accurately and readily resolving mixtures in a forensic context would have important ramifications for the forensic community by allowing otherwise uninterpretible samples to be analyzed.

§ 9 - 2 The Process of Mitochondrial DNA Analysis

§ 9 - 2.1 DNA Extraction and Amplification

The labor intensive manner of DNA extraction by Chelex™ or Phenol Chloroform has prompted the development of faster robotic platforms. The BioRobot EZ1 DNA extraction robot (Qiagen Inc., Valencia, CA), presents small to medium size forensic laboratories with a new, easy to use platform that allows 1-6 samples to be extracted simultaneously in about 15 minutes. Each sample uses a dedicated extraction channel, pipette tip and extraction cartridge as to minimize the potential for cross-contamination. This significantly cuts down

dedicated analyst time and yields DNA of significantly higher purity than existing traditional methods (Montpetit *et al.*, 2005).

Traditional mtDNA analysis of forensic evidence employs the amplification of the mitochondrial control region (D-loop). Due to significant degradation of genetic material observed when mtDNA sequencing analysis are performed, this is generally achieved in four separate amplification reactions, each amplifying a small segment of the control region (*i.e.*, HV1A, HV1B, HV2A and HV2B) (Wilson *et al.*, 1995). When amplification of these fragments fail, the analyst can further shorten the amplification target by employing a "mini-primer" set (Gabriel *et al.*, 2001b). These targets divide the traditional amplicons into two fragments each, possibly yielding results for severely degraded evidentiary material.

§ 9 - 2.2 Purification and Yield Determination of Amplified PCR Products

Following amplification, a host of methods exist for purifying amplified PCR products. In forensic mtDNA laboratories this is typically accomplished by use of spin column purification or nuclease/alkaline phosphatase treatment, (*e.g.*, ExoSAP-IT®) (Werle *et al.*, 1994; Dugan *et al.*, 2002). While these methods are effective at removing small DNA fragments, such as primers, they are less efficient at removing larger spurious amplification products. In addition, these

methods have the limitation that they cannot distinguish between target PCR amplicons and similar size spurious amplification products.

PCR amplification yield can be quantified by a number of approaches including UV spectroscopy, gel electrophoresis, or microchip capillary electrophoresis, all of which have a number of shortcomings. UV spectroscopy cannot distinguish between an amplified target and residual primers or spurious PCR amplification products resulting in the overestimation of the actual amount of amplified target DNA. Agarose “yield gels”, are moderately labor intensive and require crude comparisons of samples to a quantification ladder. Capillary electrophoresis systems offers greater accuracy and precision but requires more sample handling steps, investment in expensive dedicated equipment and higher consumable costs per sample.

Purification of DNA fragments by High Performance Liquid Chromatography (HPLC) produces suitably purified target amplicons for use in labeling reactions for DNA sequencing. Additionally, the height and area of DNA fragment peaks on a chromatographic trace is a function of the quantity of DNA detected. The use of HPLC, therefore, allows for combination of purification and quantification into a single step. This effectively eliminates the need for yield gels or other quantification methods, while also yielding information about the presence and quantity of spurious co-amplified products (Chapter 3).

In addition, HPLC can effectively be used to determine contamination levels in negative control and reagent blank samples. Due to the sensitivity of fluorescence detection based on the use of a DNA intercalating dye, picogram quantities of amplified DNA can be detected. This can eliminate the need for downstream sequencing analysis of negative controls and reagent blanks, thereby, saving valuable time and money on samples which most of the time yield no sequencing results at all.

§ 9 - 2.3 Mitochondrial DNA Characterization

To date, no method for mtDNA characterization (*i.e.*, the presence of heteroplasmy or multi-contributor mixture), prior to dye-terminator sequencing, has been validated for use in the forensic community. A variety of methods have been investigated, such as hybridization to linear arrays of sequence-specific oligonucleotides (SSO) (Reynolds *et al.*, 2000; Gabriel *et al.*, 2001a); denaturing gradient gel electrophoresis (DGGE) (Hanekamp *et al.*, 1996); single-strand conformational polymorphism (SSCP) analysis (Alonso *et al.*, 1996; Barros *et al.*, 1997; Steighner *et al.*, 1999); time-of-flight mass spectrometry (Butler *et al.*, 2001); and microarray-based analysis (Fukushima, 1999). These approaches, suffer from a number of limitations which include: the interrogation of only a subset of variant sites; cross-hybridization to non-target sequences; laborious manipulations of gels (Meyers *et al.*, 1988; Reynolds *et al.*, 2000); and interference by primer impurities (Butler *et al.*, 2001). Most critically, however,

these approaches suffer from the fact that they consume often precious forensic evidence while not necessarily providing a reliably comprehensive assessment of all possible sequence differences across the entire amplicon.

Denaturing HPLC (DHPLC) circumvents these limitations while offering the potential to characterize mtDNA amplicons in an accurate, rapid and cost-effective manner that can be easily automated. Moreover, DHPLC makes it possible to recover the assayed DNA from the column eluent at the end of an assay. The accuracy and sensitivity with which sequence variants can be detected by DHPLC can be significantly influenced by the length, GC content and other thermodynamic characteristics of the amplicon being assayed (Chapter 2). Using carefully designed assay conditions the concordance between direct DNA sequencing and comparative sequence analysis by DHPLC typically ranges from 95-100%. In some cases, DHPLC has proven to have greater sensitivity than direct DNA sequencing for the detection of minor component sequence variants.

Mitochondrial DNA characterization allows for determination of sample complexity prior to costly dye terminator sequencing and time consuming analysis. The analyst can, therefore, gain valuable information on whether a sample contains base heteroplasmy, or worse yet, is a mixture of two or more contributors. In addition to characterization, and in cases where samples contain mtDNA from more than one source, the analyst can divide the mixture into a

series of fractions in an attempt to separate individual contributors. The isolated fractions can then be subjected to direct dye-terminator sequencing analysis without the need to re-amplify the captured PCR product.

§ 9 - 2.4 Sequencing Analysis

§ 9 - 2.4.1 Single Contributor mtDNA Analysis

The profiling of human mtDNA is achieved by comparison of DNA sequence data to the revised Cambridge Reference Sequence (rCRS) (Anderson *et al.*, 1981; Andrews *et al.*, 1999). Differences are noted and reported by indicating the nucleotide position and the identity of the altered base. In cases of heteroplasmy, a base is indicated either as the combination of the two mixed bases (*e.g.,* a mixture of nucleotides "A" and "G", is reported as "R"), or as undetermined ("N").

§ 9 - 2.4.2 Mitochondrial DNA Mixture Analysis

Samples considered true mixtures by forensic interpretation standards are those which display mixed nucleotides at two or more positions. In forensic mtDNA analysis, mixed nucleotide positions are generally labeled undetermined ("N"), eliminating the nucleotide from the overall analysis. This can prevent an analyst from being able to determine the true level of discrimination between sequences that are thought to "match" (Fourney, 1998). Although strategies for separating mtDNA mixtures have been proposed (Alonso *et al.*, 1996; Hanekamp

et al., 1996; Hatsch *et al.*, 2007), all display significant obstacles for implementation by forensic laboratories.

Denaturing HPLC in combination with Linkage Phase Analysis (LPA) allow for separation and statistical analysis of mtDNA mixtures in a manner that circumvents the limitations of alternative methods. The sensitivity of DHPLC for identification and fractionation of non-identical samples and the capability of LPA to analyze peak height ratio changes of mixed nucleotides from multiple sequencing electropherograms allow for the identification of individual contributors to a mixture. Depending on the shift in peak height ratio between electropherograms obtained from different fractions, identification of individual contributors to the mixture can be achieved with high degree of statistical significance (>99%) (Chapter 6).

§ 9 - 2.4.3 Fractional Linkage Phase Analysis Resource Software (FLiPARS)

Due to the time consuming and tedious nature of sequencing analysis a software application has been developed to streamline and speed identification of mixed nucleotides and LPA of mixed mtDNA samples. The software application initially aligns all imported sequence data to the revised Cambridge Reference Sequence (rCRS) using the Needleman-Wunsch global alignment algorithm (Needleman *et al.*, 1970). It then scans each analyzed sequencing electropherogram, identifying all nucleotide positions which have a secondary

peak height above 10% of that of the major component consistent with existing interpretation standards for forensic laboratories. The identified nucleotide positions are consolidated into a table where the analyst can select which nucleotides are used for LPA. The two sequencing electropherograms which yield the greatest shift in peak height fluorescence across all mixed nucleotides positions are then used to: calculate the shift in peak height fluorescence for each mixed positions; determine the confidence of separation based on the shift in peak height fluorescence; and identify the two contributors to the mixture. In addition to this, the user interface displays the number of data points behind the statistical determination performed. Furthermore, confidence of separation is indicated for each base next to the confidence and base determination tables. A green indicator reveals ≥17% shift in peak height fluorescence and ≥99.9% confidence in separation. A yellow indicator reveals ≥6% shift in peak height fluorescence and ≥99% confidence in separation, while a red indicator reveals <6% shift in peak height fluorescence and <99% confidence in separation.

The software application has been tested on 43 blind samples using results obtained during the manually analyzed validation studies of LPA (Chapter 6). Figure 9.1 illustrates results obtained using the manual calculation method (Figure 9.1A) and FLiPARS (Figure 9.1B) for a HV2A mtDNA mixture with 8 mixed nucleotides. For all eight mixed positions, fluorescence ratio shift comparison for fractions 7 and 11 are identical, resulting in consistent determination of

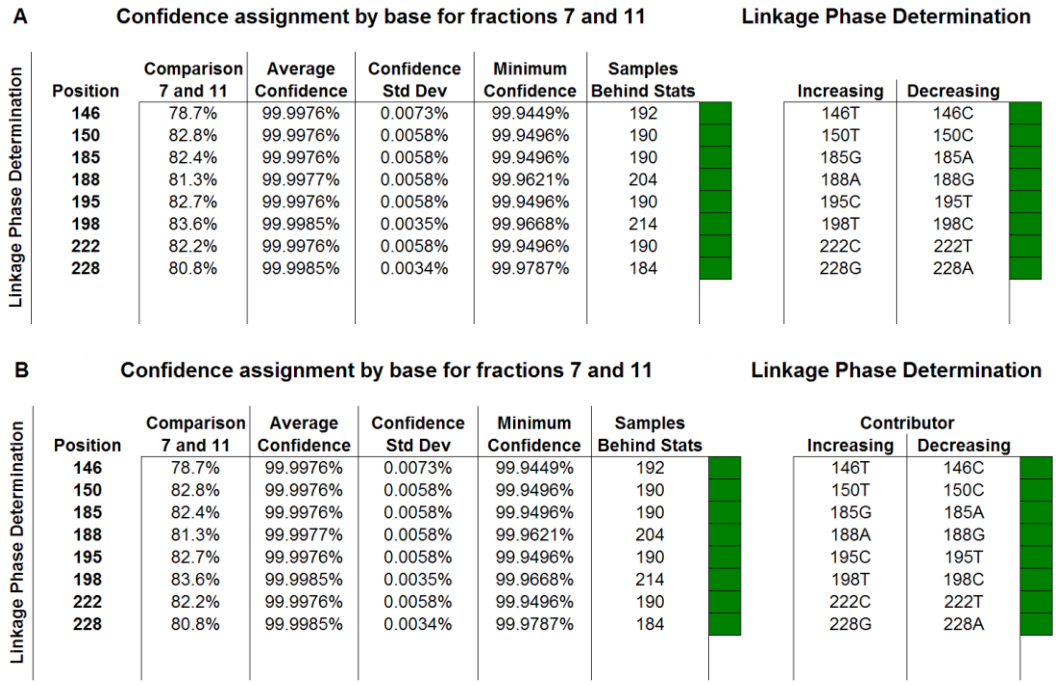


Figure 9.1: Linkage phase analysis results obtained for a two-component HV2A mtDNA mixture containing eight mixed nucleotide positions. (**A**) LPA performed using a manual calculation method. (**B**) LPA performed using an automated software application method (FLiPARS). Comparison of linkage phase results yields no differences between the two methods used and complete concordance in the contributor haplotypes identified.

contributor nucleotides and associated confidence of separation. In all mtDNA mixtures analyzed by LPA to date, 100% concordance exists between results analyzed manually and those analyzed by FLiPARS (Table 9.1).

This being said, subtle differences do exists in determinations of linkage phase between the manual calculation and those analyzed using FLiPARS. These exist purely as programming issues, the potential significance of which is not entirely known. Briefly though, sequencing of mixed templates yields two overlapping peaks at mixed-base positions which typically overlap completely. In some cases, however, the minor component peak is shifted slightly up- or downstream of the maximum fluorescence peak of the major component. FLiPARS compensates for this variability by analyzing ±3 data points (±9.28sec) away from the maximum fluorescence of the major component, in attempt to determine the maximum peak height for the minor component. However, if the minor component peak height maximum falls outside this window, the minor components true peak height will be underestimated. As the shift in minor component peak increases the larger the underestimation in peak height ratio. Any future modifications to the software will have to address this issue, by either allowing the user to manually widen the peak crest scanning range or by developing an algorithm which detects shifts in secondary component peaks and modifies the peak crest scanning range accordingly.

Table 9.1: Comparison of LPA for 47 simulated casework samples using the manual linkage phase determination and FLiPARS. Deviation in mixture separation confidence shows minimal differences between the two analysis methods.

				Max. Δ Fluorescense (Manual)		Max. Δ Fluorescense (FliPARS)		
Item	Substrate	DNA Source	Enrironmental Contaminant	Δ Fluorescence Range	Confidence Ave.	Δ Fluorescence Range	Confidence Ave.	Δ Confidence Ave.
1	Denim	Blood/Blood	None	29.3% - 41.2%	99.987%	29.3% - 40.7%	99.986%	0.001%
2	Leather	Blood/Blood	None	29.1% - 51.0%	99.988%	29.1% - 51.0%	99.988%	0.000%
3	Wood	Blood/Blood	None	28.4% - 40.7%	99.988%	28.4% - 40.2%	99.988%	0.000%
4	Carpet	Semen/Semen	None	28.4% - 51.5%	99.991%	28.4% - 51.5%	99.991%	0.000%
5	Nylon	Semen/Semen	None	27.9% - 45.1%	99.988%	27.9% - 45.1%	99.988%	0.000%
6	Carpet	Saliva/Saliva	None	19.2% - 30.4%	99.977%	19.2% - 30.4%	99.977%	0.000%
7	Leather	Saliva/Saliva	None	17.4% - 31.8%	99.974%	17.4% - 31.8%	99.974%	0.000%
8	Wallboard	Saliva/Saliva	None	21.4% - 27.0%	99.974%	21.4% - 27.0%	99.974%	0.000%
9	Wood	Saliva/Saliva	None	17.2% - 27.5%	99.965%	17.2% - 27.5%	99.965%	0.000%
10	Cotton Swab	Blood/Blood	Gasoline	29.0% - 46.7%	99.986%	29.0% - 46.7%	99.986%	0.000%
11	Cotton Swab	Blood/Blood	Motor oil	33.0% - 49.9%	99.991%	33.0% -49.9%	99.992%	0.000%
12	Cotton Swab	Blood/Blood	Detergent	28.8% - 45.8%	99.991%	28.8% - 45.8%	99.991%	0.000%
13	Cotton Swab	Blood/Blood	HAC	27.7% - 49.4%	99.989%	27.7% - 49.4%	99.989%	0.000%
14	Cotton Swab	Semen/Semen	Soil	20.4% - 23.0%	99.964%	20.0% - 23.0%	99.964%	0.000%
15	Cotton Swab	Semen/Semen	NaOH	31.0% - 48.9%	99.987%	31.0% - 48.9%	99.987%	0.000%
16	Cotton Swab	Saliva/Saliva	Gasoline	20.2% - 33.0%	99.975%	20.2% - 33.0%	99.975%	0.000%
17	Cotton Swab	Saliva/Saliva	Motor oil	24.7% - 33.3%	99.978%	24.7% - 33.3%	99.978%	0.000%
18	Cotton Swab	Saliva/Saliva	HAC	19.1% - 30.4%	99.973%	19.1% - 30.4%	99.973%	0.000%
19	Denim	Blood/Semen	None	16.3% - 20.4%	99.926%	12.9% - 18.8%	99.890%	0.036%
20	Leather	Blood/Semen	None	16.8% - 26.1%	99.934%	16.9% - 26.1%	99.934%	0.000%
21	Wood	Blood/Semen	None	19.3% - 31.8%	99.969%	19.3% - 31.8%	99.969%	0.000%
22	Carpet	Saliva/Semen	None	19.9% - 33.8%	99.978%	19.9% - 33.8%	99.978%	0.000%
23	Nylon	Saliva/Semen	None	19.2% - 32.6%	99.976%	19.2% - 32.6%	99.976%	0.000%
24	Carpet	Blood/Saliva	None	31.1% - 33.1%	99.983%	31.1% - 33.1%	99.983%	0.000%
25	Leather	Blood/Saliva	None	43.0% - 47.4%	99.994%	43.0% - 47.4%	99.994%	0.000%
26	Wallboard	Blood/Saliva	None	42.3% - 43.5%	99.993%	42.3% - 43.5%	99.993%	0.000%
27	Wood	Blood/Saliva	None	39.8% - 46.1%	99.991%	39.8% - 46.1%	99.991%	0.000%
28	Cotton Swab	Blood/Semen	Gasoline	17.3% - 24.3%	99.958%	18.2% - 23.5%	99.956%	0.001%
29	Cotton Swab	Blood/Semen	Soil	15.6% - 26.1%	99.929%	16.4% - 24.5%	99.933%	-0.004%
30	Cotton Swab	Blood/Semen	Detergent	17.0% - 27.1%	99.959%	23.7% - 26.6%	99.971%	-0.012%
31	Cotton Swab	Blood/Semen	NaOH	17.6% - 22.1%	99.954%	17.6% - 22.1%	99.954%	0.000%
32	Cotton Swab	Saliva/Semen	Gasoline	12.2% - 21.7%	99.926%	12.2% - 21.7%	99.926%	0.000%
33	Cotton Swab	Saliva/Semen	NaOH	24.8% - 28.1%	99.978%	24.8% - 28.1%	99.978%	0.000%
34	Cotton Swab	Blood/Saliva	Motor oil	41.5% - 47.2%	99.993%	41.6% - 47.2%	99.993%	0.000%
35	Cotton Swab	Blood/Saliva	Detergent	37.1% - 41.5%	99.992%	37.1% - 41.5%	99.992%	0.000%
36	Cotton Swab	Blood/Saliva	HAC	39.4% - 46.1%	99.991%	39.4% - 46.1%	99.991%	0.000%
37	None	Bone	Aged	48.7% - 61.6%	99.996%	51.9% - 61.0%	99.996%	-0.001%
38	None	Bone	Aged	11.2% - 39.1%	99.912%	10.4% - 39.4%	99.899%	0.013%
39	None	Head hair/Blood	Dyed/None	15.6% - 21.7%	99.927%	15.6% - 21.7%	99.927%	0.000%
40	None	Head hair/Blood	None	31.3% - 33.4%	99.984%	15.6% - 16.5%	99.888%	0.096%
41	None	Axial hair/Blood	None	13.7% - 24.3%	99.923%	13.7% - 24.3%	99.923%	0.000%
42	None	Pubic hair/Semen	None	28.8% - 30.7%	99.983%	28.8% - 30.7%	99.983%	0.000%
43	None	Head hair/Blood	Permed/None	11.0% - 16.1%	99.835%	11.0% - 16.1%	99.835%	0.000%
44	Cotton Swab	Vaginal swab/Semen	None	14.5% - 17.9%	99.906%	14.5% - 18.2%	99.907%	-0.001%
45	None	Head hair/Semen	None	16.1% - 18.7%	99.919%	16.2% - 18.4%	99.919%	0.000%
46	Cotton Swab	Saliva/Breat swab	None	5.7% - 25.8%	99.534%	5.7% - 25.8%	99.534%	0.000%
47	None	Head hair/Semen	None	27.3% - 44.5%	99.987%	27.3% - 44.5%	99.987%	0.000%

In addition to the essential modification to the detection of shifted peak crests, the ability to view sequencing electropherograms and modify miscalled nucleotides would be a welcomed addition to FLiPARS. This would eliminate the need for secondary electropherogram viewing/editing applications such as Sequencher™ (Gene Codes Corp, Ann Arbor, MI) or Lasergene® (DNASTAR, Inc., Madison, WI) and significantly speed analysis of mtDNA mixtures. Finally, the ability to compare sequence quality, based on optimal intensity ratio (Chapter 4) relative to a pristine reference electropherogram, would allow for a quick quality control assessment of questioned electropherograms.

§ 9 - 2.5 Streamlining of mtDNA Analysis

Obtaining an mtDNA haplotype from forensic samples is a very labor intensive process which requires multiple handling steps (Figure 9.2). Currently, mtDNA analyses require that an analyst use separate methods for PCR product purification and quantification. Determination of low level amplification in negative controls and reagent blanks currently cannot be obtained until results from dye-terminator sequencing are analyzed. Determination of whether a sample contains multiple starting templates or is site or length heteroplasmic is currently not possible until dye-terminator sequencing analyses are performed. Furthermore, low level mixtures can go unnoticed or misinterpreted as being heteroplasmic due to differential incorporation of dye-terminators. All of these

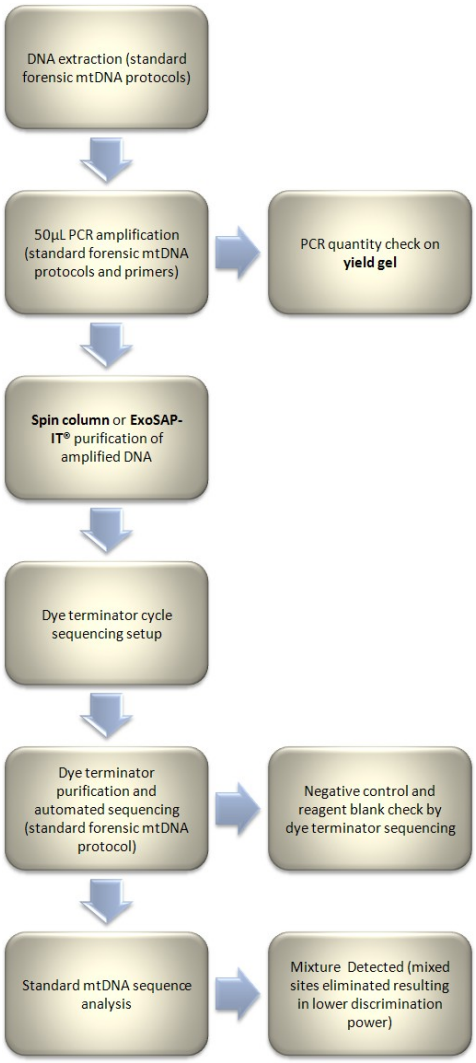


Figure 9.2: Sample process flow for traditional mtDNA dye-terminator sequencing based analysis. Two different techniques are used for sample purification and quantification. Currently no method is available for detecting the presence of mixed templates, screening negative controls and reagent blanks or identifying the haplotypes of individual contributors to mtDNA mixtures.

factors have prompted the development and implementation of mtDNA analysis by DHPLC for forensic samples.

Analysis of mtDNA amplicons using the demonstrated diversity of DHPLC applications streamlines the overall process of PCR product purification, quantification and allows for mixture detection prior to dye-terminator seuqencing (Figure 9.3). DHPLC yields highly purified amplicons, free of spurious amplification products while simultaneously providing information on the amplification yield quantity. Determination of mixed templates has traditionally required dye-terminator labeling and subsequent sequencing, however, DHPLC analysis yields immediate information on sample complexity without the need for further sample manipulation. DHPLC also adds mixture fractionation capabilities, which in conjunction with LPA allows for mtDNA mixture separation and contributor identification. Mitochondrial DNA analyses by DHPLC require only minor modifications to standard operating procedures currently employed in forensic caseworking laboratories. All DHPLC instrument components and applications have been validated in accordance with SWGDAM developmental validation guidelines (SWGDAM, 2004). These factors are essential in implementing DHPLC analysis in a forensic casework environment and for the admissibility of DHPLC and LPA in court.

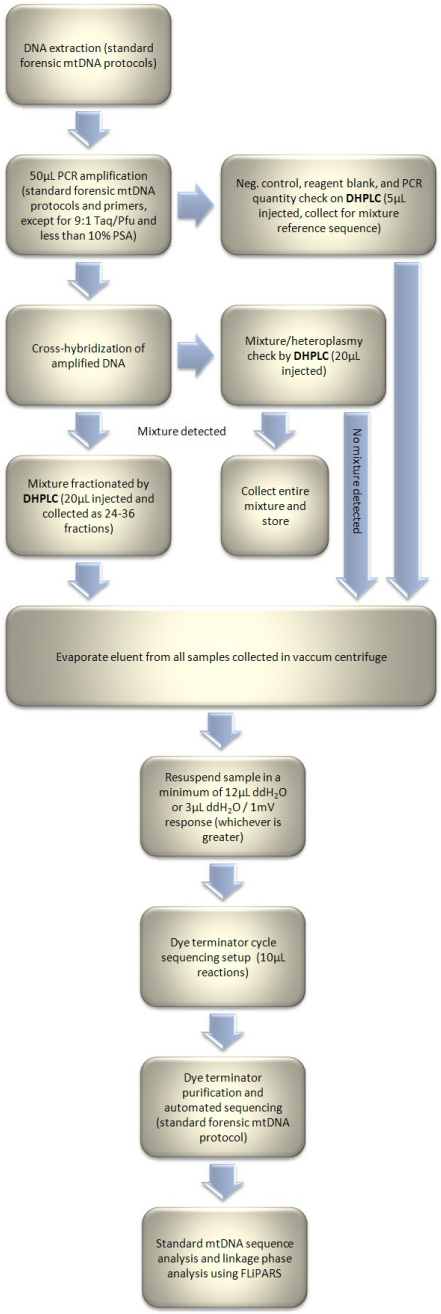


Figure 9.3: Sample process flow for mtDNA sequence analysis using DHPLC, dye-terminator sequencing and LPA. DHPLC analyses are used for sample purification and quantification in a single step. DHPLC analyses are also used for mixture identification/fractionation and to screen negative controls and reagent blanks. LPA is used to determine the linkage phase (*i.e.*, the haplotypes) of the individual contributors to mtDNA mixtures and to calculate the statistical confidence of mixture resolution for each base position.

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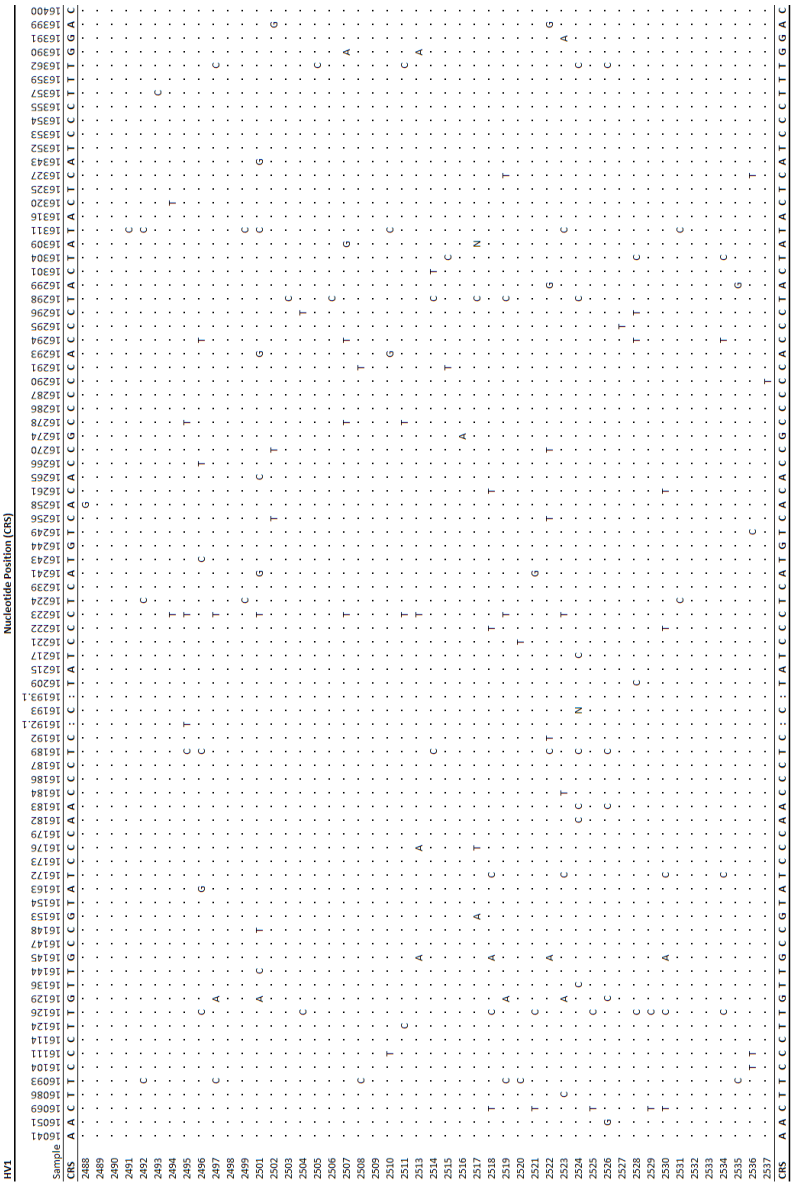
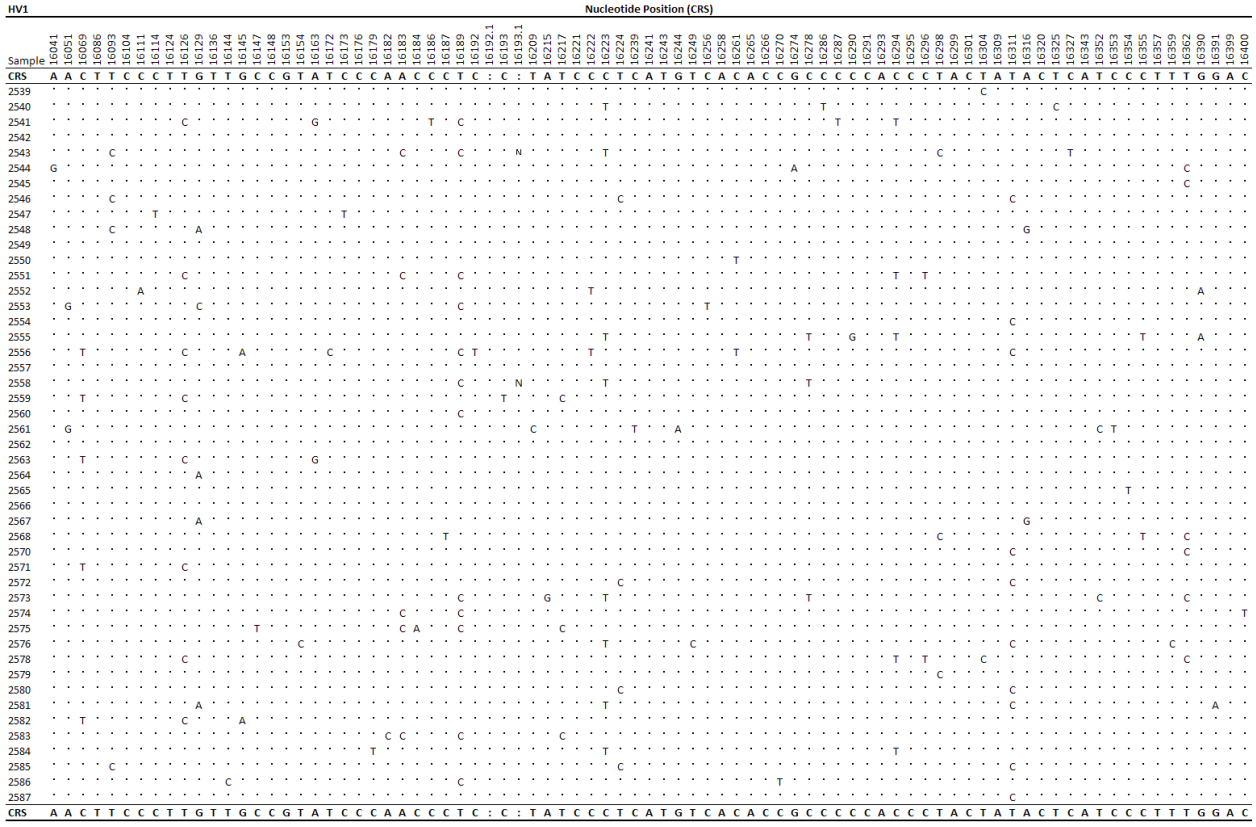
Appendices

Appendix A

Sequence polymorphisms in HV1 for the 99 samples collected and used in this study as compared to the revised Cambridge Reference Sequence (rCRS).




HV1
Nucleotide Position (CRS)
Sample
CRS
16041 16051 16069 16086 16093 16104 16111 16114 16124 16126 16129 16136 16144 16145 16147 16148 16153 16154 16163 16172 16173 16176 16179 16182 16183 16184 16186 16187 16189 16192 16192.1 16193 16193.1 16209 16215 16217 16221 16222 16223 16224 16239 16241 16243 16244 16249 16256 16258 16261 16265 16266 16270 16274 16278 16286 16287 16290 16291 16293 16294 16295 16296 16298 16299 16301 16304 16309 16311 16316 16320 16325 16327 16343 16352 16353 16354 16355 16357 16359 16362 16390 16391 16399 16400
CRS A A C T T C C C T T G T T G C C G T A T C C C A A C C C T C : C : T A T C C C T C A T G T C A C A C C G C C C C C A C C C T A C T A T A C T C A T C C C T T T G G A C

Sequence polymorphisms in HV2 for the 99 samples collected and used in this study as compared to the revised Cambridge Reference Sequence (rCRS).

HV2	Nucleotide Position (CRS)																																															
Sample	55	57	64	72	73	89	92	93	114	131	143	146	150	152	153	185	188	189	192	194	195	196	198	199	200	204	207	217	222	225	226	228	234	239	242	249	250	263	280	285	293	295	299	309	309.1	309.2	315.1	319	340
CRS	**T**	**T**	**C**	**T**	**A**	**T**	**G**	**A**	**C**	**T**	**G**	**T**	**C**	**T**	**A**	**G**	**A**	**A**	**T**	**C**	**T**	**T**	**C**	**T**	**A**	**T**	**G**	**T**	**C**	**G**	**T**	**G**	**A**	**T**	**C**	**A**	**T**	**A**	**C**	**C**	**T**	**C**	**C**	**C**	**:**	**:**	**:**	**T**	**C**
2488	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	C	C	·	·
2489	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	C	·	·
2490	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	C	·	·	·	·	·	C	·	·
2491	C	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2492	·	·	·	·	G	·	·	·	·	·	·	·	T	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2493	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2494	·	·	·	·	G	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	C	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	C	·	·
2495	·	·	·	·	G	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	A	C	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2496	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2497	·	·	·	·	G	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2498	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	C	·	·
2499	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2501	·	·	·	·	G	C	A	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	C	C	·	·
2502	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2503	·	·	T	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2504	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	C	·	·
2505	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	G	·	·	·	·	·	·	·	·	C	·	·
2506	·	·	T	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2507	·	·	·	·	G	·	·	·	·	·	A	C	·	C	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2508	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	C	·	·
2509	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	N	C	·	·
2510	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2511	·	·	·	·	G	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2513	·	·	·	·	G	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2514	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2515	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2516	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	C	·	·
2517	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2518	·	·	·	·	G	·	·	·	·	·	·	·	T	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	G	·	·	·	T	·	·	·	·	C	·	·
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2520	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	C	C	·	·
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2523	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	C	G	·	·	·	·	·	·	·	·	C	·	·
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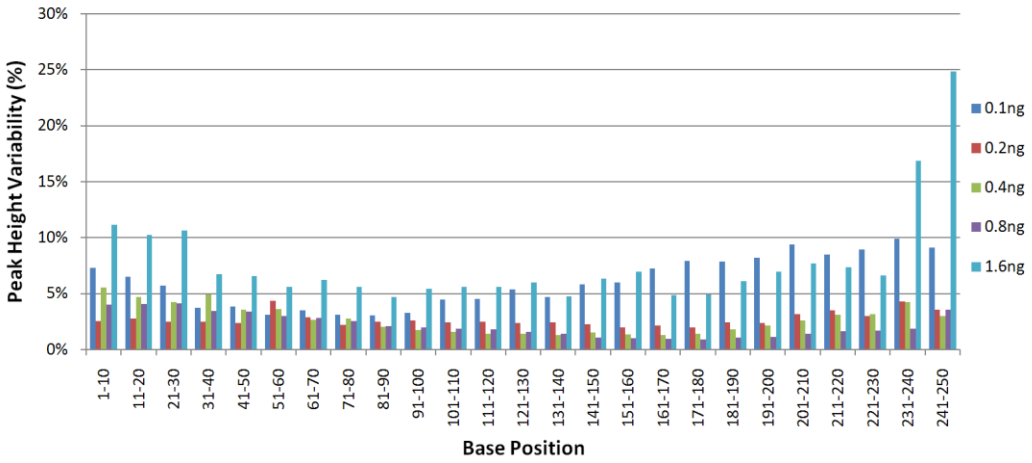
2525	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	T	·	·	C	·	C	·	·
2526	·	·	·	·	G	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	C	·	T
2527	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	C	·	·
2528	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2529	·	·	·	·	G	·	·	·	·	·	·	C	·	·	·	A	G	·	·	·	·	·	·	·	·	·	·	·	T	·	·	A	·	·	·	·	·	G	·	·	·	T	·	·	·	·	C	·	·
2530	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	G	·	·	·	T	·	·	·	·	C	·	·
2531	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	C	·	·
2532	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	C	C	·	·
2533	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	·	C	·	·
2534	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	C	C	·	·
2535	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	C	·	·
2536	·	·	·	·	G	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	T	·	·	·	·	·	·	C	·	·
2537	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	N	C	·	·
CRS	**T**	**T**	**C**	**T**	**A**	**T**	**G**	**A**	**C**	**T**	**G**	**T**	**C**	**T**	**A**	**G**	**A**	**A**	**T**	**C**	**T**	**T**	**C**	**T**	**A**	**T**	**G**	**T**	**C**	**G**	**T**	**G**	**A**	**T**	**C**	**A**	**T**	**A**	**C**	**C**	**T**	**C**	**C**	**C**	**:**	**:**	**:**	**T**	**C**

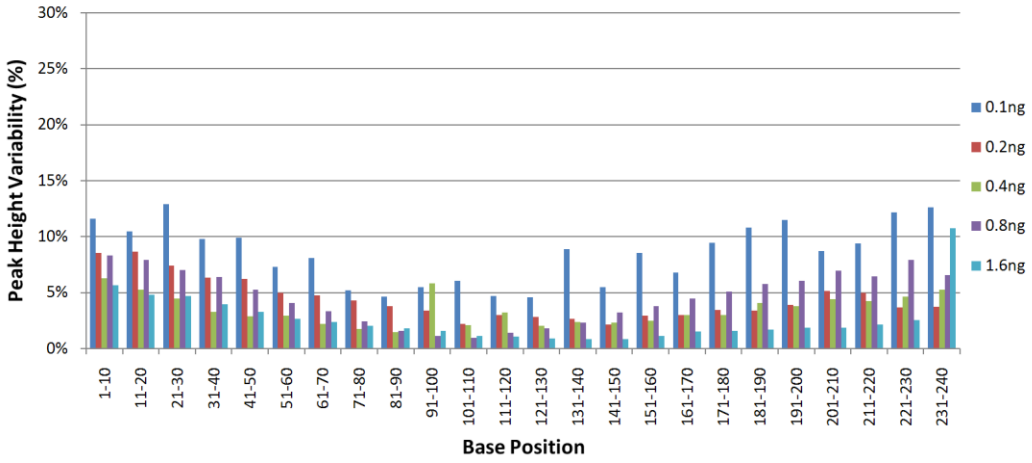
HV2 — **Nucleotide Position (CRS)**

Sample	55	57	64	72	73	89	92	93	114	131	143	146	150	152	153	185	188	189	192	194	195	196	198	199	200	204	207	217	222	225	226	228	234	239	242	249	250	263	280	285	293	295	299	309	309.1	309.2	315.1	319	340
CRS	**T**	**T**	**C**	**T**	**A**	**T**	**G**	**A**	**C**	**T**	**G**	**T**	**C**	**T**	**A**	**G**	**A**	**A**	**T**	**C**	**T**	**T**	**C**	**T**	**A**	**T**	**G**	**T**	**C**	**G**	**T**	**G**	**A**	**T**	**C**	**A**	**T**	**A**	**C**	**C**	**T**	**C**	**C**	**C**	**:**	**:**	**:**	**T**	**C**
2539	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	C	.	.
2540	.	.	.	.	G	.	.	.	.	.	A	.	.	.	.	.	.	G	C	T	C	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	C	.	.
2541	.	.	.	.	G	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
2542	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
2543	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	:	G	.	.	.	.	.	.	.	.	.	C	.	.
2544	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	G	.	.	.	.	.	.	:	.	C	C	C	.
2545	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	G	.	.	.	.	.	.	.	C	.	C	.	.
2546	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
2547	.	.	.	C	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	C	.	.
2548	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
2549	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
2550	.	.	.	.	.	C	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	C	C	.	.
2551	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	C	.	.
2552	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	C	C	.
2553	.	.	.	.	G	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	T
2554	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
2555	.	.	.	.	G	.	.	.	.	.	A	C	.	C	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	C	.	.
2556	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	G	.	.	.	T	.	.	.	.	.	C	.	.
2557	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
2558	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	A	C	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	C	.	.
2559	.	.	.	.	G	.	.	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	T	.	.	.	.	.	C	.	.
2560	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
2561	.	.	.	.	G	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
2562	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	C	.	.
2563	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	.	T	.	.	.	C	.	C	.	.
2564	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
2565	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	C	.	.
2566	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
2567	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
2568	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	:	G	.	.	.	.	.	.	.	C	.	C	.	.
2570	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	G	.	.	.	.	.	.	.	C	N	C	.	.
2571	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	.	T	.	.	.	.	.	C	.	.
2572	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.	.	.	C	C	C	.	.
2573	.	.	.	.	G	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	G	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
2574	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
2575	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	C	.	.
2576	.	.	.	.	G	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
2578	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	C	.	.
2579	.	.	.	C	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	C	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	C	.	.
2580	.	.	.	.	G	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	C	.	.
2581	.	.	.	.	G	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	C	A	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	C	C	C	.	.
2582	.	.	.	.	G	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	.	T	.	.	.	.	.	C	.	.
2583	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	:	.	.	C	.	.
2584	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	C	.	.
2585	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
2586	.	.	.	.	G	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
2587	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
CRS	**T**	**T**	**C**	**T**	**A**	**T**	**G**	**A**	**C**	**T**	**G**	**T**	**C**	**T**	**A**	**G**	**A**	**A**	**T**	**C**	**T**	**T**	**C**	**T**	**A**	**T**	**G**	**T**	**C**	**G**	**T**	**G**	**A**	**T**	**C**	**A**	**T**	**A**	**C**	**C**	**T**	**C**	**C**	**C**	**:**	**:**	**:**	**T**	**C**

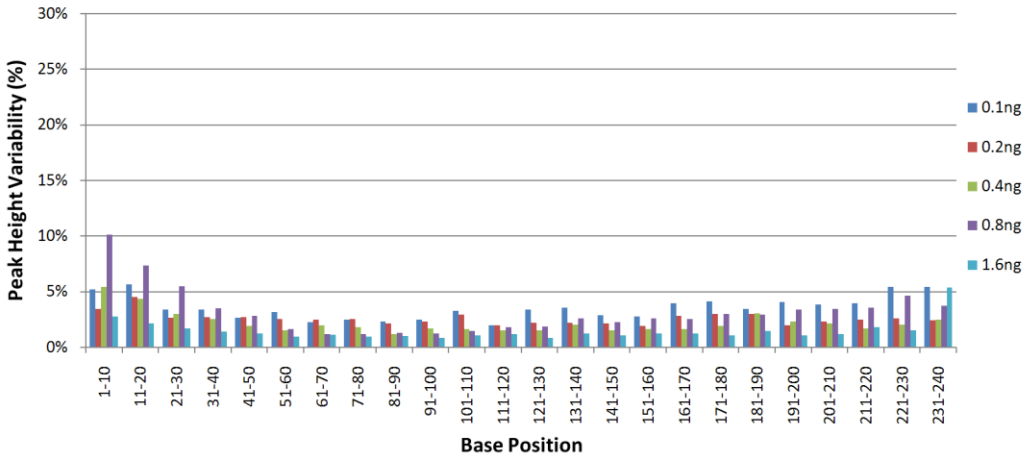
Appendix B



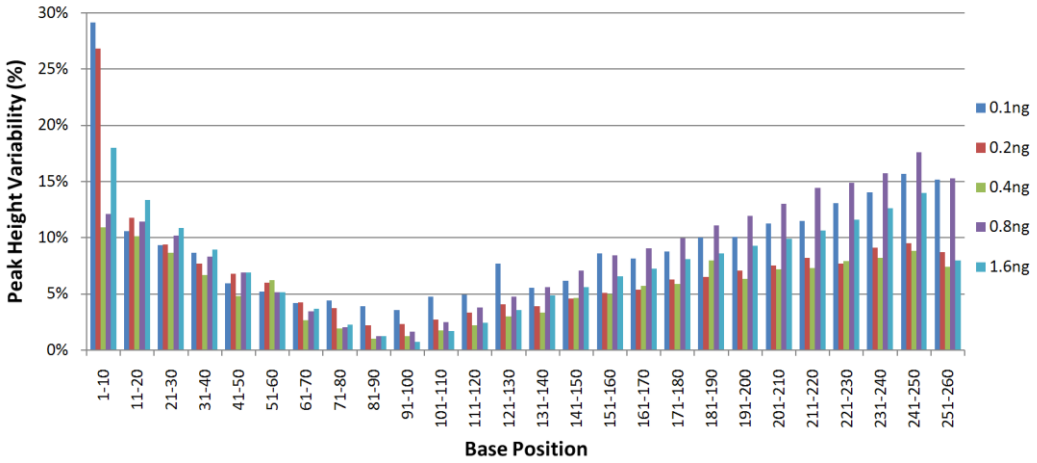
Reproducibility of electrophoretic peak heights for the mtDNA HV1A amplicon sequenced with the A1 primer (Table 2.1) . The data represent the mean peak heights ± standard deviations from five independent labeling reactions for each of five DNA input quantities.



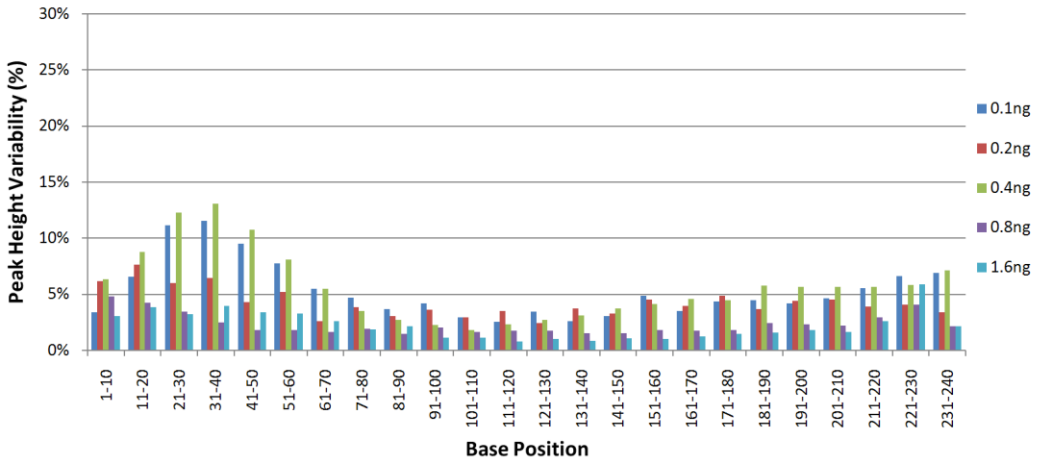
Reproducibility of electrophoretic peak heights for the mtDNA HV1A amplicon sequenced with the B2 primer (Table 2.1) . The data represent the mean peak heights ± standard deviations from five independent labeling reactions for each of five DNA input quantities.



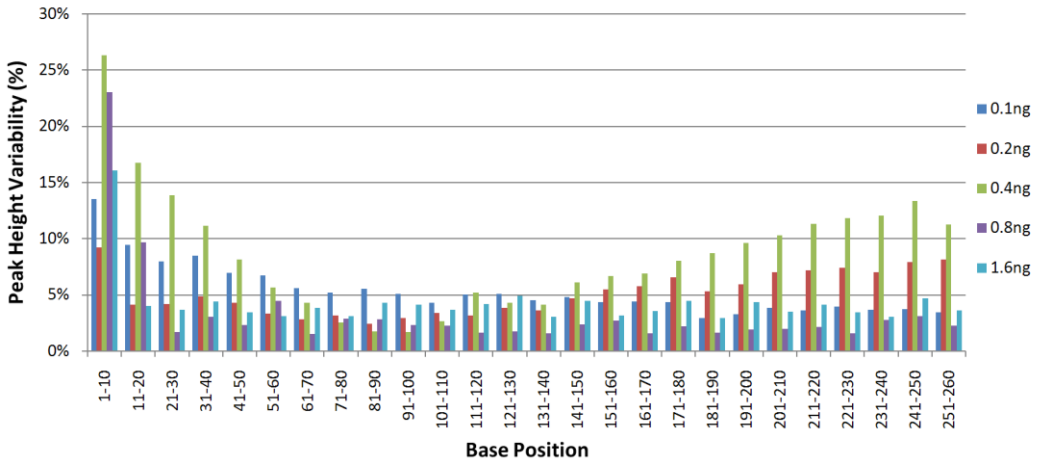
Reproducibility of electrophoretic peak heights for the mtDNA HV1B amplicon sequenced with the B1 primer (Table 2.1) . The data represent the mean peak heights ± standard deviations from five independent labeling reactions for each of five DNA input quantities.



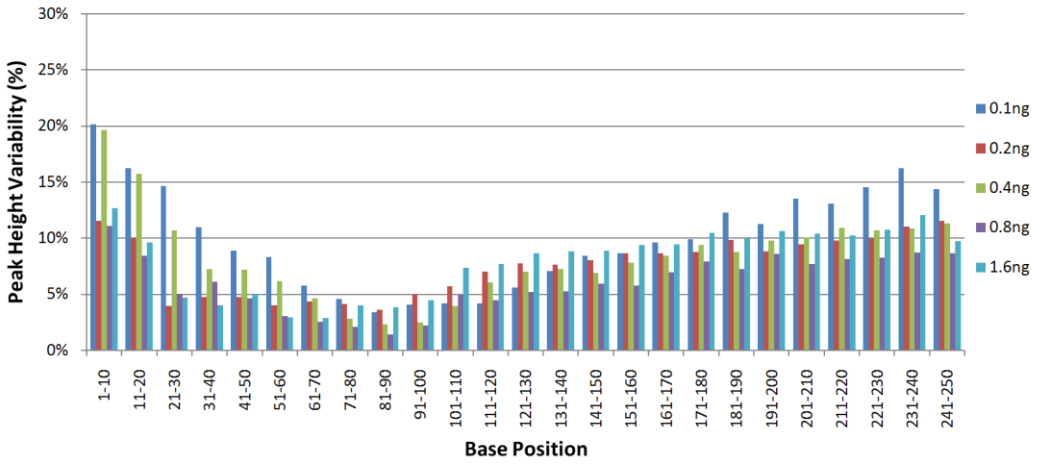
Reproducibility of electrophoretic peak heights for the mtDNA HV1B amplicon sequenced with the A2 primer (Table 2.1) . The data represent the mean peak heights ± standard deviations from five independent labeling reactions for each of five DNA input quantities.



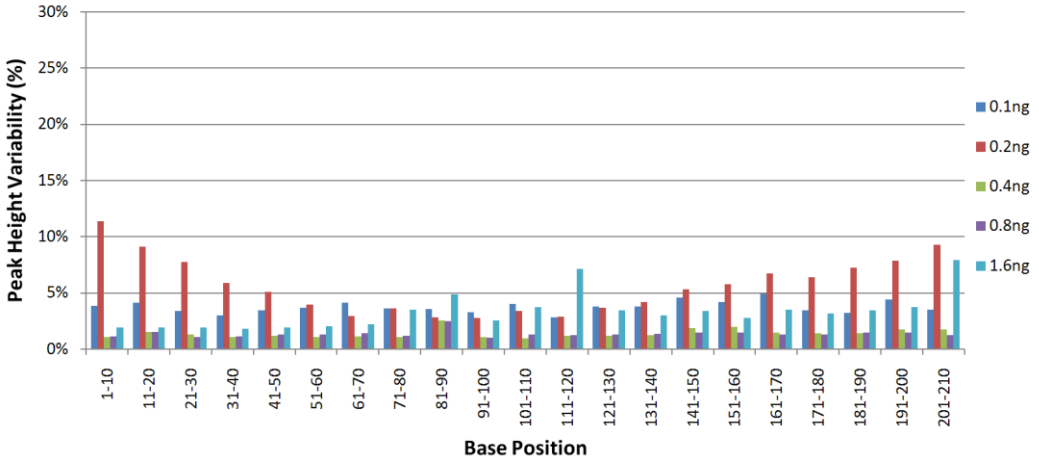
Reproducibility of electrophoretic peak heights for the mtDNA HV2A amplicon sequenced with the C1 primer (Table 2.1) . The data represent the mean peak heights ± standard deviations from five independent labeling reactions for each of five DNA input quantities.



Reproducibility of electrophoretic peak heights for the mtDNA HV2A amplicon sequenced with the D2 primer (Table 2.1) . The data represent the mean peak heights ± standard deviations from five independent labeling reactions for each of five DNA input quantities.



Reproducibility of electrophoretic peak heights for the mtDNA HV2B amplicon sequenced with the D1 primer (Table 2.1) . The data represent the mean peak heights ± standard deviations from five independent labeling reactions for each of five DNA input quantities.



Reproducibility of electrophoretic peak heights for the mtDNA HV2B amplicon sequenced with the C2 primer (Table 2.1) . The data represent the mean peak heights ± standard deviations from five independent labeling reactions for each of five DNA input quantities.